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THE BIOCHEMICAL JOURNAL

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I. THE AUTOLYSIS OF THE MUSCLE OF THE COD FISH.

By ERNEST HAROLD CALLOW.

A REPORT TO THE FOOD INVESTIGATION BOARD, DEPARTMENT OF SCIENTIFIC AND INDUSTRIAL RESEARCH, FROM THE LOW TEMPERATURE RESEARCH STATION, CAMBRIDGE

(*Received October 9th, 1924.*)

Fearon and Foster [1922] showed that ox muscle, after being frozen rapidly in brine at − 8° and then thawed, autolysed at about the same rate (in the presence of toluene) as normal muscle. On the other hand, muscle which had been frozen slowly in air at − 8° and then thawed showed a greater amount of autolytic change and a more rapid rate of autolysis. The present investigation is an extension of Fearon and Foster's work to the muscle of cod. No reference to the autolysis of cod muscle could be found in the literature. The material proved to be very difficult to manipulate and it was only after much preliminary work that satisfactory methods were worked out.

Methods.

The following procedure was finally adopted. The muscle, with connective tissue, was minced by passing twice through a very fine mincer, and then mixed thoroughly by hand; 400 g. were quickly weighed out for the digest. At the same time about 15 g. were transferred to a stoppered weighing bottle and used for determinations of total nitrogen; in each case six estimations of total nitrogen were carried out, the average being adopted. The 400 g. of mince were transferred in small quantities to a mortar and triturated with toluene until each portion was saturated, 150 cc. of toluene being used in all. The mixture was then quantitatively transferred to a sterile 2-litre flask, the total volume of suspension being made up to 2 litres with distilled water. The flask was shaken at intervals for two hours. After this, a sample was withdrawn and the flask placed in the incubator or cold room. Every 12 hours the flask was shaken vigorously, and every 24 hours two samples were withdrawn with a blunt-ended pipette delivering 24·7 cc. Each sample was placed in a stoppered 100 cc. measuring flask containing 50 cc. of a 4 % solution of trichloroacetic acid. The contents of the flask were made up to 100 cc. by addition of distilled water. The mixture was left for 24 hours and then filtered. Estimations of the nitrogen content of the filtrate (so called "soluble nitrogen") were made by Cole's alcohol distillation method [Cole, 1920]. Nitrogen determinations of the reagents were carried out and due allowance made.

In order to compare fresh and frozen muscle, a fish was bisected longitudinally. Muscle was taken from one half and minced for autolysis; this served for a control. The other half was frozen, stored, thawed and then minced for autolysis. For the processes of freezing and storing, halves of two different fish were placed together in an oiled-silk bag, so that the thickness would be the same as that of a whole fish. Thus the fish was affected neither by penetration of salt during brine freezing, nor by desiccation during air freezing and storage.

Experimental Results.

Exp. 1. *The Autolysis of Fresh Cod at* + 37°. In order to test the accuracy of the method, preliminary autolyses were carried out. The following experiment shows that the experimental error involved is quite small.

Fresh cod was minced in the usual way, and duplicate autolyses were carried out. Fig. 1 shows that such duplicates gave practically identical results, thus illustrating the accuracy of the method.

Exp. 2. *The Autolysis of Fresh Cod at* + 1°. Fresh cod was minced and put up in four similar flasks, two of which were incubated at + 37° and sampled daily. The other two flasks were kept in a room at + 1° and samples were removed at intervals of several weeks. The experiment commenced on October 5th, 1923.

Table I.

	Soluble N	
	Flask 1	Flask 2
Date examined	%	%
October 9, 1923	10·9	10·9
January 7, 1924	10·8	10·9
May 23, „	11·7	11·7
October 5, „	12·4	12·2

The results of the experiments at + 37° are shown in Fig. 1 whilst Table I shows the results at + 1°. It will be seen that the rate of autolysis at + 1° is very much reduced. From these data it was calculated that the rate of autolysis at + 37° was 223 times more rapid than at + 1°. (This gives a temperature coefficient of 4·5 for each 10°.)

Exp. 3. *The Effect of Brine-freezing at* − 19° *on the Rate of Autolysis.* Halves of two different fishes were placed together in an oiled silk bag and frozen in brine at − 19° for 2½ hours, and then stored at − 11° for 10 days. They were thawed by hanging in air at + 1° for 96 hours, then minced and autolysed at + 37° in the usual way. The other halves of the two fishes were minced without having been frozen, and incubated to give a measure of normal autolysis.

Fig. 2 shows that the brine-frozen fish gave results practically identical with those of the unfrozen control. This agrees with the results of Fearon and Foster [1922] who found that brine-freezing does not cause any appreciable change in the tissues of ox muscle.

Exp. 4. *The Effect of Air-freezing at* − 11° *on the Rate of Autolysis.* Two cod were bisected longitudinally. Half of each fish was immediately minced for a control experiment, suspensions being made in the usual way and incubated at + 37°. The other halves were placed together in an oiled-silk bag and slowly frozen by hanging in a cold chamber at − 11° for 10 days. Portions of muscle were then cut from each half-fish and placed in two separate covered

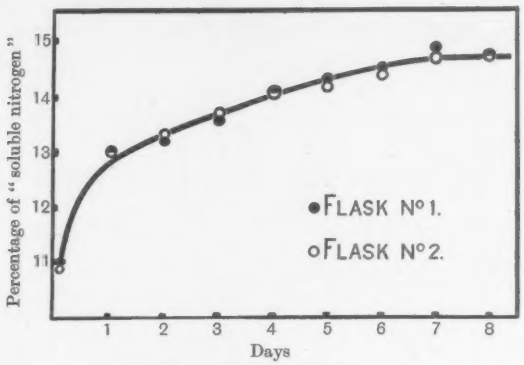

Fig. 1. Duplicate autolyses

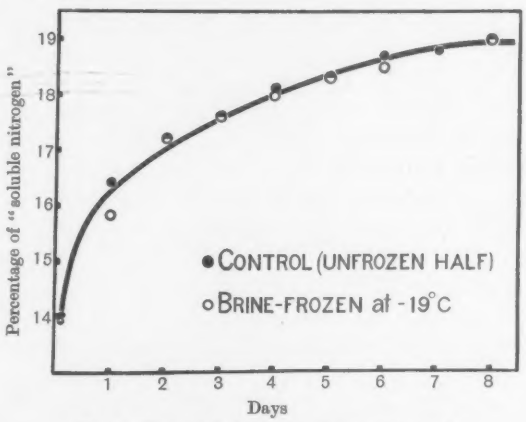

Fig. 2. The autolysis of brine-frozen cod muscle

beakers. The beakers were placed in a room at + 1° for 66 hours to ensure slow thawing. During this process liquid ("drip") separated out. The muscle was then minced and the mince and "drip" thoroughly mixed together. Suspensions were made up and autolyses were carried out at + 37° in the usual way.

The results of a typical experiment (see Fig. 3) show that in the case of air-frozen cod there is slightly more autolytic change than occurs in the unfrozen control. This result is also in agreement with Fearon and Foster's [1922] conclusions on the air-freezing of ox muscle.

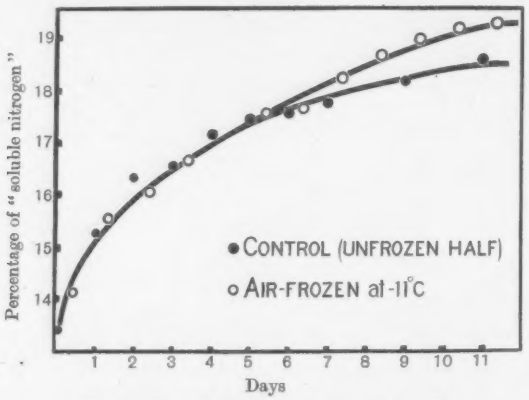

Fig. 3. The autolysis of air-frozen cod muscle

Exp. 5. *The Autolysis of "Drip."* "Drip," that is the fluid exuded by slowly frozen tissues on thawing, was collected from a cod frozen in air at − 19°. 100 cc. of this fluid were mixed with 37·5 cc. of toluene and the volume made up to 500 cc. with distilled water. The mixture was incubated at + 37° and samples were withdrawn daily and analysed. It was found that "drip" contained a higher initial percentage of soluble N (27·9 %) than cod muscle, and that the *increase* of soluble N (to 36·4 %) during the course of autolysis was also greater than in the case of muscle.

Discussion.

Previous work [Stiles, 1922; Richardson and Scherubel, 1908; Kallert, 1923] on the freezing and thawing of animal tissues has shown that the minimum of alteration is obtained when the tissues are frozen rapidly and thawed slowly. The reason for this is that during freezing ice crystals separate out—the slower the freezing the larger the ice crystals formed and *vice versa*.

Obviously the ideal method is to freeze so rapidly that the size of the ice crystals is a minimum. Brine freezing at low temperatures appears to give a rate rapid enough for most practical purposes.

It has been observed that, on thawing frozen tissues, the water formed from the ice is slowly reabsorbed by the tissues [Kallert, 1923; Richardson and Scherubel, 1908.]

Even after complete thawing has been accomplished, this process of absorption still goes on. In the present investigation this fact was borne in mind,

the muscle tissues being given ample opportunity to reach equilibrium before the autolyses were carried out.

The results of this investigation show that air-frozen cod suffered a much greater destruction of its tissues than brine-frozen cod. Cod muscle frozen rapidly in brine at – 19° gave no "drip" and presented a perfectly normal appearance on thawing, whereas cod frozen slowly in air at – 19° and – 11° always "dripped" on thawing and felt noticeably flabbier. The volume of "drip" which could be collected in such cases was of the order of 10 % of the weight of the fish. Also, rapidly frozen cod muscle autolysed at the same rate as the unfrozen control whilst with slowly frozen cod muscle there was a slight but appreciable increase in the amount and rate of autolysis. Thus for cod muscle as for other animal tissues the best method of preservation is to freeze rapidly.

Experiments carried out at + 1° showed only slight autolysis even after 12 months. This is of importance as it indicates that no appreciable changes are likely to occur in the proteins of *frozen* cod stored for prolonged periods at low temperatures such as – 11°.

Note on a Protein of Cod Muscle.

When "drip" was incubated at + 37° with toluene, a coagulum was observed after 24 hours. It consisted chiefly of protein. The following experiments with cod muscle offer further evidence for the existence of a protein which coagulates at + 37°.

1. Air-frozen cod muscle whilst still in the frozen condition was minced, suspended in a 5 % solution of $MgSO_4 7H_2O$ and left over night. The suspension was then filtered and the filtrate gently heated to + 37·5° when a faint opalescence was observed. Another experiment on the same filtrate gave an opalescence at + 38°.

2. The following experiment proved that the presence of $MgSO_4 7H_2O$ had no appreciable effect on these results. Shavings of air-frozen cod were ground up in a mortar with sand and distilled water. The filtrate from this extraction also contained a protein which commenced to coagulate at + 36°. This experiment was repeated with fresh cod with the same result.

Summary.

1. At + 37° autolysis of cod muscle was practically complete after seven to eight days but at + 1° autolysis proceeded very slowly and even after 12 months the changes were quite slight.

2. Cod frozen rapidly (in brine at – 19°) presented a normal appearance on thawing, and on autolysis at + 37° gave results identical with those of the unfrozen control.

3. On thawing cod which had been frozen slowly (in air at – 11°), a fluid ("drip") separated out. Autolysis of the thawed tissue with the appropriate amount of "drip" proceeded at a slightly greater rate than that of the unfrozen control.

4. "Drip" was found to contain a higher initial percentage of soluble N and to autolyse to a greater extent than normal cod muscle.

5. Cod muscle was found to contain a protein which coagulates at + 37°.

My thanks are due to Professor F. G. Hopkins, F.R.S., for the interest he has shown in the investigation.

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II. THE PREPARATION AND PROPERTIES OF PURIFIED OLEIC ACID AND SOME OF ITS SALTS.

By ARTHUR LAPWORTH, LEONORE KLETZ PEARSON and (in part) EDWARD NEVILLE MOTTRAM.

From the University, Manchester.

(*Received November 12th, 1924.*)

Notwithstanding the large amount of work which has been done on the chemistry of oleic acid the published methods for preparing it leave much to be desired. The descriptions given of the compound itself inspire so little confidence that even the most recent edition of the Handbook of Lewkowitsch [1921] quotes melting points for oleic acid varying from 6·5° to 16°, although Kirschner's conclusion [1912] that the acid is dimorphous, is accepted. Edmed [1898] by recrystallisation of Kahlbaum's oleic acid from alcohol obtained a sample of the acid which was stated to melt at 13–15°. Partington [1911] by crystallising another specimen obtained from Kahlbaum, and probably one of inferior grade, could not obtain a product melting higher than 6·5°; Kirschner [1912] by following much the same procedure obtained samples of the acid which showed dimorphism and therefore two different melting points. The present authors originally hoped to devise an improved method for the estimation of oleic acid in mixtures such as are obtained by hydrolysis of natural fats and oils, but soon found that it would be necessary to study the question of preparing and characterising pure oleic acid almost *ab initio*.

During the course of their studies, now extending over some years, the authors have been forced to retrace their steps many times, but they believe that they can now with advantage place some of their results on record and indicate with considerable confidence the probable margins of experimental error. Having tried nearly all the published devices for purifying oleic acid and numerous combinations and permutations of these and others, they have worked out a process by which oleic acid containing not more than 1 % of impurity can, with practice, be obtained with considerable certainty.

Separation of salts of higher fatty acids by recrystallisation.

The authors have latterly confined their experiments to the acids obtained by hydrolysis of olive oil, in which it is known that palmitic acid is the main saturated constituent. It is not intended to discuss this point in the present paper, but it may be stated that the authors have never detected the presence

of any saturated acid higher than palmitic acid in samples of oleic acid made from olive oil by the process recommended, so that stearic acid, for example, if present in the crude acid, was mainly eliminated as lead salt in the first stages of purification. In addition to oleic acid, palmitic acid and any acids which are easily eliminated owing to the sparing solubility of their lead salts, there are present linolic acid and perhaps others still more highly unsaturated. [Compare Hehner and Mitchell, 1896.]

Various metallic salts of oleic acid, linolic acid and palmitic acid with barium, lead, lithium and sodium have been examined by the present authors especially with regard to their suitability for the purpose of separating the acids from one another. It is unnecessary to go into the large mass of experimental detail and it will be sufficient to say that when a mixture of palmitic acid and oleic acid was converted into barium, lead, lithium or sodium salts and these were fractionated by crystallisation, the palmitate was invariably found in larger proportion in the solid than in the dissolved portions. This behaviour is quite consistent with solubility curves for solid solutions of certain types and renders it highly improbable that any recrystallisation process applied to these salts can yield pure oleates[1].

It has been suggested by C. W. Moore [1919] that the use of salts of bivalent metals for separating oleic acid from other acids is to be avoided owing to the possibility of double salts, such for example as barium oleo-linolate, being formed. The objection would doubtless hold good if barium oleo-linolate, for example, were less readily soluble than barium oleate, but as the mixed salts invariably, in the experience of the present authors, exhibit a much higher degree of solubility than the pure oleates, the difficulty is not a serious one from the theoretical point of view, and in actual practice the purification of barium oleate from barium linolate by recrystallisation is comparatively easy.

Properties of mixed lead salts of oleic and other fatty acids.

The separation of palmitic acid and other saturated fatty acids from oleic acid is as a rule effected by making use of the greater solubility of lead oleate as compared with palmitate, in ether, benzene or petroleum, usually the first. It is widely known that this method is imperfect but it is perhaps not generally realised that it is very rough indeed. The solubility of lead palmitate in ether, benzene and other solvents is not diminished as might be anticipated, but is in fact greatly increased by the presence of lead oleate, linolate, etc. Unless, therefore, care is exercised to remove, with the lead palmitate, a large proportion of the oleate itself, the oleic acid prepared by the lead salt process may contain 5 % or even more of palmitic acid. On freezing the solution, however, the lead oleate which then separates out carries down with it a large proportion of the remaining lead palmitate and finally an oleic acid can be

[1] The forms of the freezing point "curves" of mixtures of oleic and palmitic acid (compare Fig. 1, p. 15) also indicate that any complete purification of the former by a recrystallisation process applied to a mixture of the free acids would not be practicable.

isolated from the mother liquor which does not contain more than about 2 % of palmitic acid, which is the smallest proportion of palmitic acid which it has yet been found practicable to obtain in any sample of oleic acid purified by a process involving crystallisation only.

It must be emphasised too, that the solubility of lead oleate, for example, in ether, varies considerably not only with the quantity of other salts present but apparently also with the quantity of alcohol and of water present and also with time. A clear solution of freshly precipitated, crude lead oleate in ether, if left for some days or weeks in a closely stoppered vessel, gradually deposits much of the salt in solid form and the deposit is then comparatively very sparingly soluble in ether. The cause of this phenomenon is obscure.

If a fresh ethereal solution of the mixed lead salts from olive oil be filtered at ordinary temperatures and then cooled, amorphous lead oleate mixed with palmitate tends to be deposited above − 10°, but below that temperature the deposit tends to become crystalline and on warming to ordinary temperatures does not always wholly redissolve. The property which lead oleate has of becoming crystalline below − 10° may be taken advantage of, if desired, to remove the bulk of the more highly unsaturated acids, as a preliminary in preparing oleic acid from olive oil, using ether as a solvent. In this case the ethereal solution remaining after the bulk of the palmitate has been separated by filtration at ordinary temperatures is slowly cooled to − 15° with constant agitation, and as soon as the deposit has become granular[1] the whole may be filtered through a Buchner funnel with the aid of a pump; the physical condition of the crystalline deposit is so well fitted for rapid filtration that when using several hundred grams of ether the solid may be secured without difficulty or serious loss of solvent even if no special precautions are taken to cool the funnel beforehand. In this way the bulk of the lead salts of the more highly unsaturated acids may be removed and subsequent processes of purification to some extent facilitated.

Barium oleate.

Farnsteiner [1901] first drew attention to the fact that barium oleate, although nearly insoluble in hot benzene containing absolute ethyl alcohol, dissolves if a very little water is present, and comes out on cooling in lustrous needles or long laminae. He claimed that a method for the quantitative separation of oleic acid from more highly unsaturated fatty acids could be based on this, as the barium salts of these remained dissolved; this conclusion however was disputed by Lewkowitsch [1903] who stated that, in presence of these acids, barium oleate is soluble in the medium. The experience of the present authors agrees with that of Lewkowitsch, and in another paper data will be furnished which fully substantiate his statement. The barium salt of oleic acid may be made use of, however, for the isolation of highly purified oleic acid from a mixture which contains a large proportion of that acid—such as that from

[1] Repeated warming and cooling is sometimes necessary to obtain a granular material.

olive oil. The authors find, however, that several great advantages are to be gained by substituting amyl alcohol for ethyl alcohol. When ethyl alcohol is used there is danger of the water, present or added, causing the alcohol to separate in diluted form as a distinct phase; with amyl alcohol on the other hand, if there is excess of water, it is the excess of water which remains as the separate phase and has no ill effect. Toluene was used in the later experiments rather than benzene as it is less volatile; the mixture of toluene and amyl alcohol is readily recovered with but little change in composition, by distillation in a current of steam, and used again as solvent. A moist mixture of equal volumes of toluene and amyl alcohol is recommended for rapid purification of barium oleate, but toluene with 10 % of amyl alcohol may be used.

Dry barium oleate is practically insoluble in benzene or toluene even on boiling. If a little of this salt is heated with either of these liquids in a test tube and a drop of water is added the powdery particles of the salt swell and soon the whole forms an opaque jelly and remains as a jelly when cool. On subsequent addition of one drop of amyl or ethyl alcohol and again heating, a thin, slightly opalescent solution is at once formed, and this on cooling deposits the salt in shimmering flat needles. The salt is soluble in a hot mixture of nearly dry amyl alcohol and benzene (or toluene) but on cooling comes down as a powder, which has no crystalline appearance unless a little water is first added.

The solubility of pure barium oleate in a moist mixture of equal volumes of amyl alcohol and benzene was found to be 0·219–0·224 g. per 100 cc. at 12°, determined about 12 hours after cooling a warm saturated solution to that temperature; it may be considerably less if the time of standing be prolonged. This, although small, is nearly fifteen times as large as that found by Farnsteiner for his solvent mixture of benzene and moist ethyl alcohol at 11° [1901]. This greater solubility extends to the other salts present in crude barium oleate, and the fact presents a definite practical advantage when the separation of barium linolate and similar salts from barium oleate is concerned, for these impurities in the oleate are in consequence more rapidly removed on recrystallisation[1]. For rapid elimination of the salts of the more highly unsaturated acids the filtrations are best carried out within an hour or two of cooling to 12°, but it is even better to cool no lower than to 20° and filter at once.

The process which the authors now use for preparing the most highly purified oleic acid is as follows.

Isolation of oleic acid from olive oil.

(With Edward Neville Mottram.)

Olive oil (400 g.) is heated on the steam-bath under a reflux condenser for 3½–4 hours with spirit (800 g.) and water (200 cc.) containing excess of sodium

[1] When Farnsteiner's mixed solvent is used for the latter purpose, it is almost impossible wholly to remove the more soluble barium salts by a limited number of crystallisations, unless the filtrations are carried out at temperatures not lower than 38–40°.

hydroxide. The whole is then cooled and its alkalinity nearly but not quite destroyed by addition of acetic acid, using phenolphthalein as indicator. A strong aqueous solution of lead acetate is slowly added while the whole is agitated, when a precipitate is formed and adheres to the walls of the containing vessel; the addition of lead acetate is discontinued when no further precipitation ensues. The supernatant liquid is poured off and worked up to recover alcohol, the lead precipitate is fairly well drained and then dissolved in toluene (1 litre) with the aid of a steam-bath. The toluene solution is now heated for some time with solid dehydrated lead acetate (50 g.)[1]; this dissolves, but on cooling to ordinary temperature separates once more together with some 20 % of the whole quantity of the other lead salts originally present (crude lead "palmitate")[2].

After cooling the solution to ordinary temperatures and filtering from a residue of about 75–80 g. of insoluble lead salts, the clear toluene solution is shaken with excess of cold, dilute hydrochloric acid until no more lead chloride is formed on removing a portion of the upper solution and agitating with dilute hydrochloric acid. It is not difficult to separate the toluene solution of fatty acids from the aqueous layer in spite of the presence of finely divided lead chloride, and filtration through several layers of filter paper in a large Buchner funnel may be resorted to if necessary. The clear solution is subsequently agitated with water until quite free from hydrochloric acid, separated, and the solvent removed by distillation, finally under diminished pressure (15–20 mm.) on the water-bath.

The acids are now subjected to fractional distillation under a pressure not exceeding 2–3 mm.[3] when the middle fractions are used to prepare the purest oleic acid, care being taken to reject a larger quantity of the earlier fractions than of the later ones, as the main impurity, which the operation is used to eliminate, is the relatively volatile palmitic acid[4]. The earlier portions of the

[1] The lead acetate is used to effect the complete conversion of any remaining sodium salts into lead salts and it also has the effect of clearing the toluene solution and rendering the crude lead "palmitate" easy to filter off. The excess is easily recovered by washing this crude lead "palmitate" with water.

[2] By means of a freezing mixture more lead salt, rich in palmitate, separates out, and the more of this which can be removed, the simpler does the subsequent removal of palmitic acid by fractional distillation become. The removal of 50 % of all the dissolved salts has yielded the best results, but in this case ether is much to be preferred to toluene as solvent. Some lead oleate, contaminated with much palmitate, may be obtained by extracting this crude lead "palmitate" with toluene, benzene or ether, and may be reconverted into free acid, and added to the next batch of oleic acid, before subjecting the latter to the lead salt separation process.

[3] The authors used a very simple fractionating column 15 inches in length and about 1½ inches in diameter made by the "Scientific Glass-blowing Company, Manchester," which has proved very suitable for fractional distillations under reduced pressure, and was fused to the flask. The required reduction in pressure was attained by means of a mercury vapour pump of the Völlmer type in series with an ordinary Sprengel-Bunsen water pump.

[4] The authors collected their distillates in the first fractionation in two main parts, in order of passing over, representing respectively about (I), one-third and (II) one-half of the total quantity of acid, a dark orange residue (III) representing rather more than one-sixth of the

distillate set to a nearly solid mass (mixed crystals of oleic and palmitic acid) on cooling and it is of interest to observe that the authors did not discover any solid saturated fatty acids in the least volatile portions of the material although, when the lead salt separation as a preliminary was omitted, such acids separated out from the residue in the flask[1].

The fractions selected are now treated in the following manner. A mixture of equal volumes of toluene and amyl alcohol, weighing roughly ten times as much as the oleic acid to be worked up, is heated to water-bath temperature with a pinch of phenolphthalein and rather more powdered crystallised barium hydroxide than is required to neutralise the oleic acid. The oleic acid is added to the hot suspension, and the whole shaken. Barium oleate is formed very quickly and, when the whole appears permanently suffused with the colour of alkaline phenolphthalein, it is allowed to settle. The nearly clear supernatant liquid is poured off while still hot and allowed to cool, when the barium oleate separates, usually in beautifully crystalline form; it is filtered off, washed with toluene and drained.

For the purification of the barium oleate, the drained salt is recrystallised from about two-and-a-half times its weight of a hot mixture of amyl alcohol and toluene in equal volumes, with addition on each occasion of a few drops of water, the process being repeated until 100 g. of the filtrate (filtered after remaining in the cold for about twelve hours at about 12°) yields not more than 0·21 g. of residue on evaporation. From two to five recrystallisations are usually necessary and the salt should be washed with toluene on each occasion[2]. The salt requires now only to be redissolved, separated from insoluble impurities by filtration, and finally freed from adherent solvent by exposure to air or in a vacuum (if desired, after washing with ether). About 90–100 g. of barium oleate containing 2 or 3 % of barium palmitate is thus obtained. Before conversion into free acid the salt must be freed entirely from all odour of amyl alcohol and then suspended in cold water containing

original quantity of acid remaining in the distilling flask. Fraction (II) was refractionated and the corresponding fraction of this worked up for the purest oleic acid. The fractions not used for conversion into barium salt will yield more good oleic acid by repeating the whole process *ab initio*.

On a large scale, with the incomparably more efficient fractionating columns available in technical work, middle fractions could no doubt be obtained in one operation even purer than those obtained by the authors in two operations and representing a much larger proportion of the crude acid distilled.

[1] It is very doubtful whether these contained stearic acid, which has been stated by some workers not to be present in oleic acid from olive oil. It may be that they are acids higher than stearic acid, in which case it may prove satisfactory on the large scale to omit the lead salt separation process and trust to the fractionating process alone to effect the removal of all saturated acids; in view of the conflicting statements before mentioned, however, and the comparison of the results actually obtained on the small scale with and without the lead salt separation process, the present authors are not yet prepared to recommend the omission.

[2] The purification of the barium oleate from the more soluble barium salts is effected in a smaller number of recrystallisations if the temperature is not allowed to fall too low and if the filtrations are carried out as soon as possible after the selected temperature has been reached. A temperature of 20° is suitable, but if too high, clogging of the filter may occur.

a slight excess of 10 % hydrochloric acid. In the course of some hours, with frequent shaking, much salt is decomposed and if the whole be filtered through a layer of filter paper with the aid of a pump any unchanged salt may be recovered. The oily layer in the filtrate is separated and shaken repeatedly with dilute hydrochloric acid and then with water until free from barium. This mode of working, if somewhat tedious, prevents any contamination of the oleic acid with solvents or contained impurities.

The washed oleic acid is collected and after being freed from water as far as possible by mechanical means is transferred to a distilling flask, dried in a vacuum with the aid of very gentle warmth and fractionated under a pressure of not more than 2–3 mm. The great bulk distils almost constantly, usually between 170° and 175° when using a Völlmer glass mercury vapour-jet pump in series with a Sprengel water pump. About 53 g. are obtained from 100 g. of fractionated oleic acid (crude).

The bulk of the distillate should set readily, on cooling below 11°, to an ice-like mass, the thermometer rising to and remaining practically constant at not lower than 12° and sometimes as high as 12·8°[1].

Oleic acid obtained by the authors when using the above process of purification contains about 2 % of palmitic acid, which reduces its setting point by only about 0·5°. It also contains, however, small quantities of other impurities of unknown character, which are probably products of decomposition of oleic acid by heat and which further affect its setting point by several tenths of a degree; these may for the most part be removed however by draining on porous earthenware, in a vacuum, at 8–10°, while the palmitic acid cannot be so removed.

Properties of oleic acid.

Oleic acid, as Kirschner [1912] has shown, is dimorphous. This dimorphism is exhibited only by moderately pure oleic acid, and is often manifested during solidification, by the setting in of a slow rise in temperature at some stage after the rapid rise after supercooling to the steady setting point of the low melting modification. A constant higher setting point never seems to be reached in practice, however, and this is doubtless because change from the first to the second solid modification is very slow.

In other cases the oleic acid sets to an icy mass and only after some hours displays the phenomenon shown so well in the photograph given in Kirschner's paper, namely, the development of white, opaque sub-spherical aggregates of minute needles, into which the whole is ultimately transformed.

The highest melting point observed for the more stable form was 17·45°, the sample being one which, with an original setting point of 12·04°, had been left for some months during the winter. Another sample, with an original

[1] Several per cent. of palmitic acid do not greatly affect the melting point of oleic acid, but a high melting point is a fairly good guarantee that the more highly unsaturated acids and other impurities are absent.

setting point of 12·8°, was found to melt at 17° after remaining some days in the solid state.

The best samples of oleic acid made by the present authors, if slowly cooled while in the fluid condition, began to deposit crystals when the temperature had fallen to about some point between 12·3° and 12·8° and the temperature then rose quickly to a maximum, usually remaining then almost constant until the whole was nearly solid[1]. It was very seldom that this supercooling was greater than 0·65–0·7°, but Kirschner was able to keep his oleic acid at 8° for some hours and could find no temperature of spontaneous crystallisation. Kirschner, however, prepared his oleic acid by the repeated partial solidification and centrifugalisation of Kahlbaum's purest preparation of oleic acid, some samples of which he found to deposit a solid which had the characters of elaidic acid; his acid was therefore apparently saturated with elaidic acid and may have contained other fatty acids capable of forming isomorphous mixtures with oleic acid without greatly affecting its melting point.

Some further observations on the properties of purified oleic acid.

Mr Edwin Richardson has observed that when freshly distilled oleic acid contained in a glass vessel is exposed to diffused daylight for about twelve hours a small but definite depression of the setting point is often observed, amounting to about 0·07–0·1°. After this initial period further change is very slow and even when the acid is exposed to air, or when dry air is bubbled through it at ordinary temperatures for five hours, the setting point is lowered by only one or two hundredths of a degree. On the other hand, the setting point of a sample which had been left in a closely-stoppered glass bottle for about a year had fallen by about 1°.

Saturation of dry, purified oleic acid with water leads to a depression in the setting point of about 0·35° (mean of 4 concordant observations). Water is less soluble in oleic acid at 12° than at 25°, so that on cooling oleic acid saturated with water, considerable cloudiness is observed.

First setting points of mixtures of oleic and palmitic acids.

A weighed sample, usually amounting to 5–10 g. of each of numerous specimens of drained and undrained oleic acid prepared by processes not quite so efficient as the one above described, was placed in a thin glass tube which was surrounded by another which provided an air-space separating the former from the cooling bath of water. The bulb of the thermometer[2] was immersed

[1] This temperature is what hereafter is termed the "first setting point." When any marked change in temperature from the first setting point was observed, during the solidification of oleic acid containing less than 6 or 7 % of palmitic acid, it was a rise, due to the appearance of the second and higher melting modification.

[2] The temperatures given in this section were taken with a standardised thermometer capable of being read to 0·01° and are given to the nearest 0·05°.

in the acid, which was stirred with a platinum spiral. The rate of cooling in any series of observations was kept as nearly as possible constant and the first maximum temperature reached after solidification set in was recorded as the "first setting point," the observation being repeated until consistent results were obtained with a steady rate of cooling. Small weighed pellets of palmitic acid were added, dissolved by warming and stirring and new observations made.

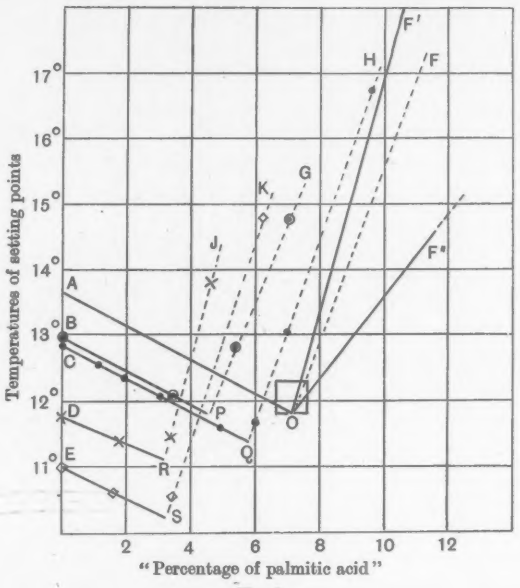

Fig. 1.

The general character of the results obtained will readily be understood by reference to Fig. 1, which exhibits actual observations made with four different samples of fairly high grade oleic acid. The *percentage of palmitic acid* was in each case the weight of palmitic acid added divided by the total weight of material present. One of these, giving the points on *BPG*, was selected because it was practically free from secondary impurities; another, *CQH*, because it was the last sample examined and a specially large number of observations was made with it in order finally to decide whether a real break occurred. The other two, namely *DRJ* and *ESK*, illustrate the behaviour of somewhat less pure specimens. The descending branches (reading from left to right) of the setting point "curves" had a slope practically independent of the amount of impurity in the samples examined. The slope of descent was invariably nearly 0·25° for each additional 1 % of palmitic acid. The steeper branches of "curves" actually traced in practice are

indicated by interrupted lines, as the slopes of these varied somewhat with the speed of cooling adopted and consequently were more or less fortuitous, but served to determine the approximate position of the "breaks."

The temperature at which the break occurs with each sample would of course always be the same if only oleic acid and palmitic acid were present. The fact that this break-temperature usually appears below 11·8° is due to very small quantities of oily secondary impurities; these are probably of low molecular weight and therefore very effective in depressing the melting point, but apparently with little influence on the iodine number or on the horizontal displacements of the "breaks" as these were scarcely altered after draining the acid; thus the undrained sample which gave the points on *ESK*, gave those on *DRJ* after draining.

Consideration of the numerous results obtained makes it possible to conclude that the lower melting modification of pure oleic acid forms with palmitic acid a series of solid solutions, the liquidus "curve"[1] of which is given approximately by the line *AO* (Figs. 1 and 2). Beyond *O*, which corresponds with a total of 7·2 % of palmitic acid and a temperature of 11·8°, another series of solid solutions (liquidus = *OF'*)[2] appears which is doubtless that corresponding with the liquidus curve given by Carlinfanti and Levi-Malvano [1909]. The curve as shown by these authors is the one marked *BC* in Fig. 2, and is such as would be obtained by use of an oleic acid containing some 7 % of palmitic acid and also of other impurities, resulting altogether in a regular displacement of the true curve *AOC* towards the left. The approximate positions of the points on *OC* have been deduced by making regular proportional displacements of *BC* towards the right.

The lowest possible temperature for *O* (Fig. 1) was fixed by that of the break observed with the sample which gave the results indicated by the lines *BP* and *PG* (Fig. 1). This sample had been repeatedly remelted and redrained until its first setting point was constant at 12·95°; it probably contained only minute traces of anything other than oleic and palmitic acids and its "iodine number" was 88·0, corresponding with a content of 2·2 % of palmitic acid.

The true percentage of palmitic acid in the pure eutectic, in other words the position of the point *O* with respect to the left-hand margin of the figure,

[1] These are termed "curves" although within the range of Fig. 1 they did not differ appreciably from straight lines.

[2] When the right-hand branches of the curves were worked out by observations of "first setting points" made with the usual speed of cooling adopted by the authors, namely about 0·2–1·0° per minute, they had slopes roughly parallel with *OF*. This has a slope between that of the true liquidus curve and that of the solidus curve, but considerably nearer to the former. The true liquidus curve is given approximately by the full line *OF'*, which is nearly parallel with the curves which the authors obtained, by plotting the temperatures at which solid disappeared on warming or by plotting "first setting points" when an extremely low rate of cooling was adopted. The slope of *OF* agrees satisfactorily with that of the left-hand extremity of the liquidus curve found by Carlinfanti and Levi-Malvano [1909, p. 371]. In order to make these features clearer the short full line *OF''* has been added to the figure; this line indicates the slope (not of course the position) of the left-hand extremity of the solidus curve as given by Carlinfanti and Levi-Malvano.

was decided by reference to the preceding data for the specimen giving the "curve" *BPG*, taken in conjunction with data worked out with special care for a second sample of oleic acid which showed a break of 3·1 % of palmitic acid (added) and which had an "iodine number" of 86·00 (mean of three observations: 86·01, 86·09, 85·76)[1]. The former places *O* at 6·8 % of palmitic acid and the latter at 7·55 %, and *O* has therefore been set about half-way between these points, namely at 7·2 %, with the reservation that this estimate may be inaccurate by 0·5 %.

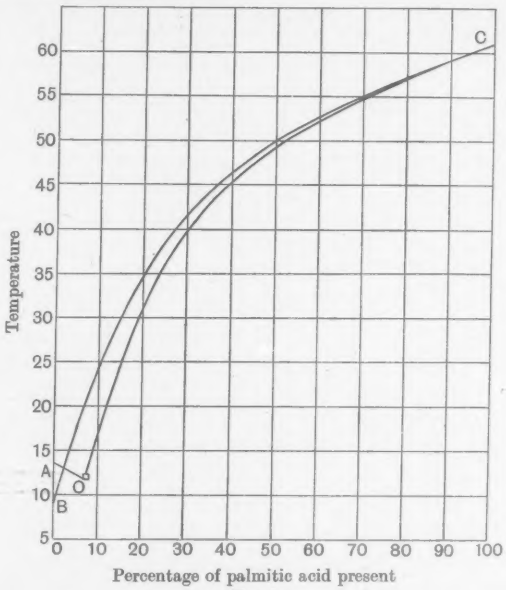

Fig. 2.

The authors believe that any changes in the position of the "curves" *A*, *E*, *B* revealed by more accurate work in the future are not likely to do more than slightly raise the lines *AO* and *OF* (Fig. 1) parallel with themselves or

[1] The "iodine numbers" were determined by a modification of the familiar bromine absorption method which was found to give very consistent results. The authors were not wholly satisfied with the bare assumption that the low iodine numbers of the purified samples of acid were due to the presence of palmitic acid, and they therefore oxidised two different samples of acid with alkaline permanganate and isolated from the products that part of it which was soluble in petroleum and non-volatile in steam; it was found to be nearly pure palmitic acid.

Thus: Sample I. Iodine number, 86, corresponding with 95·55 % of oleic acid. Palmitic acid isolated and weighed 3·3 %, making a total 98·85 %.

Sample II. Iodine number 87·2, corresponding with 96·88 % of oleic acid. Palmitic acid isolated, 1·6 %, making a total of 98·48 %.

Taking into consideration the great practical difficulty of isolating the whole of the palmitic acid present in the samples, the authors believe that the results support their view that the "iodine numbers" were trustworthy to 1 part in 100 and that their best samples of oleic acid were free from all but traces of acids of more highly-unsaturated character.

displace them slightly to the right or left. The small square indicates what the authors consider to represent the probable limits of any future alterations in the position of the common point *O*.

The work described in the present paper was carried out under the auspices of the Oils and Fats Committee of the Food Investigation Board, who defrayed a large part of the expenses incurred.

One of us (L. K. P.) is indebted to the Department of Scientific and Industrial Research for the receipt of a personal grant and another (E. N. M.) for a maintenance allowance as a student receiving training in research.

The authors have pleasure in stating that the work was undertaken at the suggestion of Mr John Allan, whom they have frequently consulted during its progress.

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III. THE NITROPRUSSIDE REACTION OF BACTERIA.

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(*Received November 21st, 1924.*)

In the present investigation an attempt has been made to obtain information as to the presence of glutathione in bacterial cells, as distinct from the culture medium, by means of the nitroprusside test [Hopkins, 1921]. Large quantities of various pure cultures of bacteria were grown in glucose broth, or on agar plates, and then freed from the culture medium. The proteins were removed by precipitation and the filtrate was investigated for the nitroprusside reaction by a quantitative method. Absence of the nitroprusside reaction indicates the absence of glutathione, but the positive reaction cannot be taken as final proof of the presence of this substance until more evidence is available.

Preliminary experiments on the nitroprusside reaction of bacteria were performed by one of us (A. B. C.) in 1922. At that time no quantitative method had been described, so the following method was devised. Pure cultures of bacteria were grown in glucose broth in Roux bottles. The broth was removed by centrifugalisation and the bacteria were washed twice with distilled water, and dried in a vacuum desiccator. The dried mass was powdered finely, and a weighed amount was mixed with 10 cc. of a saturated solution of ammonium sulphate and filtered. All the bacteria examined gave a clear colourless filtrate. In each case 0·2 cc. of a freshly prepared 5 % solution of sodium nitroprusside and 0·2 cc. of strong ammonia were added to the filtrate. The colour obtained was at once compared with a deep pink standard colour. If the filtrate gave a pink colour paler than that of the standard, a larger weight of bacteria was used, and in this way the weight of each organism required to give the standard colour was obtained.

Table I. *Preliminary Experiments.*

	Weight of dried organism which gave standard colour under standard conditions g.
B. pyocyaneus	0·04
B. prodigiosus	0·08
B. proteus	0·1
B. alcaligenes	0·1
B. fluorescens	0·1
Staphylococcus aureus	negative
Sarcina aurantiaca	,,

The results of this experiment show that *Staphylococcus aureus* and *Sarcina aurantiaca* were negative, that is to say, that even large weights failed to give a pink as deep as the standard colour. On the other hand, the other bacteria all gave strong nitroprusside reactions. If the substance responsible for this reaction is glutathione, the actual percentage calculated as dry weight works out as 0·5 % for *B. pyocyaneus*, 0·25 % for *B. prodigiosus* and 0·2 % for the other three positive bacteria. These figures were obtained by matching the standard colour with a known solution of reduced glutathione, which had been treated with nitroprusside as described above.

Since the completion of the preliminary experiments, Tunnicliffe[1] has described a quantitative method for the estimation of glutathione in muscle. Tunnicliffe found that the SH group of reduced glutathione is quantitatively oxidised by iodine. He precipitated the proteins of muscle with trichloroacetic acid and titrated the filtrate with *N*/100 iodine, using the nitroprusside test as an outside indicator. We tried to apply this method to bacteria. *B. alcaligenes* was grown for fifteen hours on forty large agar plates, then carefully scraped off, and ground in a mortar with distilled water until an even emulsion was obtained. 1 cc. was placed in a small weighed centrifuge tube, centrifugalised to remove excess water, then dried in a vacuum desiccator and weighed. In this way, the dry weight of bacteria in each cc. of emulsion was obtained. Another measured volume of the bacterial emulsion (usually 10 or 20 cc.) was precipitated with an equal volume of 20 % trichloroacetic acid. The mixture was ground in a mortar and then quantitatively transferred to a Buchner funnel, and filtered on a pump. The precipitate was removed, washed with distilled water, and again filtered. The two filtrates were added together and titrated with 0·00888 *N* iodine from a micro-burette, using the nitroprusside test as an outside indicator. Other bacteria were not so easy to manipulate, and solid trichloroacetic acid had to be used for precipitation. With *B. proteus* and *Sarcina aurantiaca*, filtration was so slow that it had to be abandoned in favour of centrifugalisation, the deposit being washed with distilled water, and the washings added to the liquid to be titrated. Several organisms, such as *S. acidi lactici* and the anaerobe *B. sporogenes* were grown on glucose broth instead of on agar plates, in order to obtain heavier growth. In all cases young cultures were used. The dry weight of the bacteria used for titration, calculated from the weight of organisms in 1 cc. of emulsion, is given with the results to show that negative results were not due to lack of material. From the titration result, the number of milli-equivalents of iodine required for 1 g. of each organism (dry weight) was calculated.

The results show that *B. alcaligenes* and *B. proteus* gave a strong nitroprusside reaction, compared with which the other bacteria examined were practically negative. This means that a drop of the trichloroacetic acid filtrate of the latter gave no nitroprusside reaction. If, however, 5 or 10 cc. of the filtrate was examined, most of the bacteria gave a very faint nitroprusside

[1] In the press.

Table II. *Trichloroacetic Acid Method.*

	Dry weight of bacteria used g.	Number of milli-equivalents of iodine for 1 g. bacteria (dry weight)
B. alcaligenes	1·5072	0·019
"	1·5885	0·018
B. proteus	0·3577	0·018
"	0·5890	0·020
B. coli	0·7260	0
B. sporogenes	0·462	0
Streptococcus acidi lactici	0·9240	0
Sarcina aurantiaca	0·2639	0
Staphylococcus aureus	1·1235	0
Timothy Grass bacillus	0·7935	0

reaction. Since very small amounts of glutathione give the reaction, the amount of this substance present in these bacteria must be considered practically negligible. Even when 10 cc. of the filtrate were examined, the Timothy Grass bacillus gave no trace of the nitroprusside reaction, and it is possible that the fatty envelope of this organism may have prevented the escape of any glutathione that might be in the cell. Possibly also in the case of the other negative bacteria glutathione might be present in the cells and yet escape detection, but this is unlikely since the organisms were ground up with trichloroacetic acid. The negative result obtained for the anaerobe *B. sporogenes* agrees with McLeod and Gordon's observation that the anaerobe *B. welchii*, when scraped off the surface of a solid medium, gave no nitroprusside reaction [McLeod and Gordon, 1924].

Three strongly pigmented bacteria, *B. pyocyaneus*, *B. fluorescens* and *B. prodigiosus*, could not be investigated by Tunnicliffe's method because in each case the pigment interfered with the titration. The earlier experiments had shown that, when these organisms were precipitated with saturated ammonium sulphate, a colourless filtrate was obtained, so that this method of precipitation was now used in conjunction with the iodine titration. Control experiments showed that the sample of ammonium sulphate used contained no substance capable of reacting with iodine. However, an objection to the use of this reagent is that, although the saturated solution is acid, after being ground up with bacteria the resulting mass is not acid enough to ensure the stability of the SH group. Tunnicliffe and Dixon [1923] showed that the SH group autoxidises far more rapidly in neutral than in acid solution. In order to lessen the possibility of such a decomposition, a trace of sulphuric acid was added to the ammonium sulphate filtrate. Even with this precaution, however, this method has given us slightly lower results than the trichloroacetic acid method. A comparison of the two methods gave the following results for *B. alcaligenes*:

Trichloroacetic acid method: 0·020 milli-equivalents of iodine per g. of dried bacteria.

Ammonium sulphate method: 0·016 milli-equivalents of iodine per g. of dried bacteria.

In default of a better method, the highly-pigmented bacteria were examined by this method.

Table III. *Saturated Ammonium Sulphate Method.*

	Dry weight of bacteria used g.	Number of milli-equivalents of iodine per g. of bacteria (dry weight)
B. pyocyaneus	0·8586	0·019
B. prodigiosus	0·5475	0·018
B. fluorescens	0·6556	0·014
B. coli	0·6928	0

The results show that the three highly-pigmented bacteria, like *B. alcaligenes* and *B. proteus*, contain a considerable amount of the substance responsible for the nitroprusside test. If this substance is glutathione, the percentages can be worked out by Tunnicliffe's experimental data. He found that 1 cc. of *N*/100 iodine reacts with 0·0025 g. reduced glutathione. This gives a value of about 0·47 % for the glutathione content of *B. alcaligenes*, *B. proteus*, *B. pyocyaneus* and *B. prodigiosus*, and 0·35 % for *B. fluorescens*. It will be noticed that these results agree fairly well with those of the preliminary experiments. In each case the same five bacteria gave strong nitroprusside tests, and *Staphylococcus* and *Sarcina* were negative.

At this point we should like to emphasise the fact that at present we have not enough evidence to state that the nitroprusside reaction of bacteria is due to the presence of glutathione, since certain substances other than glutathione also give this reaction. Besides various substances which do not contain sulphur, cysteine, which like glutathione contains the SH group, gives the nitroprusside reaction. Also H_2S in dilute solution gives the pinkish-purple colour that is characteristic of reduced glutathione, although with stronger solutions a deep blue colour is obtained. For this reason, any nitroprusside test performed in the presence of H_2S cannot be considered as evidence of the presence of glutathione.

McLeod and Gordon[1] tested broth cultures of various bacteria for the nitroprusside reaction, but, except in the case of *B. welchii*, they do not appear to have freed the bacteria from the culture medium before performing the test. An obvious objection to this method is that broth cultures of many bacteria contain large quantities of H_2S. However, McLeod and Gordon consider that their positive results are not due to this substance, because the reaction was little, or not at all, reduced by prolonged boiling.

Our method of obtaining bacteria free from H_2S was, firstly, to separate them from the culture medium. Another precaution adopted was the addition of glucose to the broth, as we found that this appeared to lessen or inhibit the production of H_2S by certain aerobes. Moreover, in our experiments, after precipitation of the proteins, the strongly acid filtrate was allowed to boil

[1] Practically all the experiments described in this paper had been completed before the publication of McLeod and Gordon's paper.

under reduced pressure on the water pump, and in this way any trace of H_2S would be removed. Proof that this was actually the case is supplied by the results obtained for the anaerobe *B. sporogenes*. This organism formed large quantities of H_2S when grown on glucose broth, and traces of H_2S still remained even after the bacteria had been freed from broth and washed. Yet the trichloroacetic acid filtrate of this organism gave no trace of nitroprusside reaction. Further proof of this point was obtained in the following way. *B. alcaligenes* was grown on agar, the bacteria were scraped off the plates, precipitated with trichloroacetic acid and filtered. Two equal portions of the filtrate were placed in small flasks. Through one of these (Flask A) a stream of nitrogen was bubbled for two hours, the other (Flask B) being placed beside it as a control. Both solutions were then titrated with iodine with the following result.

Titration result.

Flask A (after nitrogen)	...	2·66 cc. 0·00888 *N* iodine.
Flask B (control)	... ...	2·62 „ 0·00888 *N* „

If the nitroprusside test had been due to H_2S Flask A would have given a lower result, as some of the H_2S would have been removed by bubbling nitrogen through this acid solution. Since the results are practically identical, H_2S cannot be responsible for the nitroprusside reaction of *B. alcaligenes*.

Discussion of Results.

It may be noted that the presence of a substance capable of giving the nitroprusside reaction bears no relation to the oxygen uptake [Callow, 1924] of the organisms investigated. Thus, although *S. acidi lactici* and the anaerobe *B. sporogenes*, which lack the nitroprusside substance, both show practically no oxygen uptake, yet *Staphylococcus aureus* and *Sarcina aurantiaca*, which also lack the substance, both take up large quantities of oxygen. An interesting point is that all the bacteria that give the nitroprusside reaction, under the conditions described, show particularly luxuriant growth on ordinary laboratory media; for instance, on the surface of an agar plate, the growth of these bacteria is considerably heavier than in the case of the other bacteria investigated.

McLeod and Gordon [1924] consider that their results show that the positive reaction obtained with certain *broth cultures* is merely due to the fact that these bacteria reduce the oxidised glutathione which they state is present in the uninoculated broth. They further state that they consider that bacteria are not themselves capable of producing glutathione, or substances allied to it that react with nitroprusside. We cannot yet state whether or not bacteria contain glutathione, but our results show that certain bacteria undoubtedly contain a substance which gives the nitroprusside reaction, and which, like glutathione, is soluble in trichloroacetic acid and can be oxidised by iodine. (Further experiments on this point are in progress and will shortly be

published.) On the other hand, we can state that our negative bacteria either lack glutathione, or else their glutathione escapes detection by the methods used in this investigation.

SUMMARY.

1. Bacteria were grown in bulk and separated from the culture medium. The proteins were precipitated with trichloroacetic acid or saturated ammonium sulphate, and the filtrate was examined for the nitroprusside reaction by a quantitative method.

2. *B. coli*, *B. sporogenes*, *Sarcina aurantiaca*, *Staphylococcus aureus*, *Streptococcus acidi lactici* and the Timothy Grass bacillus gave practically no nitroprusside reaction and can therefore be considered to lack glutathione, unless this substance is present in the cells and escapes detection by the methods used.

3. *B. alcaligenes*, *B. proteus*, *B. pyocyaneus*, *B. prodigiosus* and *B. fluorescens* gave strong nitroprusside reactions, the iodine equivalents of which are given.

4. The substance which is responsible for the nitroprusside reaction has not yet been identified, but experiments with *B. alcaligenes* show that it is not H_2S.

In conclusion we gladly take this opportunity of thanking Professor F. Gowland Hopkins, F.R.S., for his encouragement and valuable criticism during the progress of this work. One of us (A. B. C.) is indebted to the Medical Research Council for a whole-time grant.

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IV. THE DIAZO REACTION IN URINE.

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The extensive literature on the "diazo reaction" in urine leaves many problems still unsolved. [For literature *v.* Neubauer-Huppert, 1913.] Ever since it was found that some urines gave an orange or red colour in an alkaline solution of diazotised sulphanilic acid there have been periodic attempts to isolate the substance responsible for the reaction, but so far none of these has been quite successful. Only two colour-giving substances have, as far as the writer can find, been isolated from urine and identified. The first was urocanic acid isolated by Jaffé [1874] and later found by Siegfried [1898] in dogs' urine—the dogs probably presenting a rare anomaly of metabolism. The second substance was histidine, isolated in small amount by Engeland [1908] from 40 litres of human urine. Again, in 1922 the writer [Hunter, 1922, 2] isolated 0·1 g. of histidine as monohydrochloride from a litre of measles urine in which it was estimated that there was 0·31 g. present.

Many suggestions have been made as to the cause of the diazo reaction in urine. Clemens [quoted by von Noorden, 1907] claims to have isolated the sodium salt of the substance responsible for the reaction. Others assume it to be due to chromoxy-proteic acids or to ill-defined members of this group of substances [Neubauer-Huppert, 1913]. Weiss [1920] is emphatic that urochromogen is responsible for the diazo reaction, whilst Hermanns and Sachs [1921] consider that an oxidation product of tyrosine is responsible for the reaction in tuberculous patients and hydroxyindoleacetic acid was indicated in a urine from a patient suffering from cancer of the liver.

Ehrlich first applied the reaction to urine but his procedure has been many times modified and this adds to the difficulty of interpreting the findings by his method. Ehrlich's diazo reagent consisted of a mixture of two solutions [Ehrlich, 1883]:

(*a*) 5 g. of sulphanilic acid with 50 cc. conc. HCl made to 1 l. with water.

(*b*) 5 g. of sodium nitrite made to 1 l. with water.

The solution for carrying out the test was made by mixing 50 cc. of solution (*a*) with 1 cc. of solution (*b*).

In performing the test [Erhlich, 1884] a quantity of urine was taken and an equal quantity of reagent added. If a colour was produced at this stage —what was called a "primary colour reaction"—it indicated the presence of

bilirubin. Strong ammonia was then added, and if the test was positive a "secondary colour reaction" was obtained and a red foam on shaking, regarded as of special significance in typhoid, measles, certain types of tuberculosis and other disorders.

There are thus two distinct types of diazo reaction according to Ehrlich—the primary colour reaction in acid solution and the secondary colour reaction in alkaline solution—parallel with the fact that aromatic amines couple with diazonium salts in acid solution and phenols in alkaline solution.

The primary colour reaction of Ehrlich appears to be very specific for bilirubin and this test has recently been used by van Bergh and others [McNee, 1923] in blood serum to distinguish certain types of jaundice. The diazo reaction in acid solution will not be further dealt with in this paper as there has never been any doubt about the substance responsible for it. It is the substances in urine which give a colour with diazonium salts in alkaline solution which have been wholly responsible for differences of opinion in the literature on the diazo reaction in urine; and it is with the diazo reaction in alkaline solution that the writer here purposes to deal. Hitherto only one type of diazo reaction in alkaline solution has been recognised, but, as the sequel will show, there are what may be regarded as two quite distinct types of diazo reaction in alkaline solution.

The writer has had the advantage of using a much more satisfactory diazo reagent than that used by the older investigators on this subject, namely that of Koessler and Hanke [1919], and has reviewed elsewhere the main substances which give a colour with the reagent [Hunter, 1922, 1]. These include phenols, iminazoles, certain purines, tyrosine, sulphides and ammonium salts. The reagent is thus very unspecific but no less so than the older reagents. With careful handling of the solution to be tested the non-specificity of the reagent is perhaps more of an advantage than a disadvantage as it permits the use of a very delicate reagent in the estimation of a wide variety of substances. Its reaction in urine will be considered here only from a qualitative aspect.

The diazo reagent, according to Koessler and Hanke [1919], consists of two stock solutions:

(*a*) 9·0 g. of sulphanilic acid with 90 cc. of 37 % HCl made to 1 l. with water.

(*b*) 50·0 g. of 90 % sodium nitrite made to 1 l. with water.

The *reagent* is prepared by placing in a 50 cc. volumetric flask 1·5 cc. of solution (*a*) along with 1·5 cc. of solution (*b*). After 5 minutes 6·0 cc. of solution (*b*) are added and after another 5 minutes the flask is filled to the 50 cc. mark with cold water. It is recommended by the authors to keep the flask immersed in a freezing mixture, and if kept cold this reagent can be used for at least 24 hours.

A 1·1 % solution of pure anhydrous sodium carbonate is used as alkali.

When the reagent is used for quantitative measurements the sodium

carbonate solution and the diazo reagent are first mixed and then the solution to be tested is added.

This preliminary mixing of sodium carbonate and reagent has several advantages, and the mixture remains perfectly clear and colourless for about 30 minutes, so that all the colour developed is due to the test solution added.

The use of sodium carbonate as alkali is also important. Ehrlich used ammonia as alkali although it gives an orange yellow colour with the reagent in a few seconds. On the other hand, Pauly [1904] used solid diazobenzene sulphonate and sodium carbonate, but this mixture quickly turns orange.

Another important point emphasised by Koessler and Hanke is the order in which the solutions are used. The acid reagent has an excess of nitrous acid and may partially deaminise the substance to be tested before it has time to couple in the solution subsequently made alkaline. To prevent such a deamination it is necessary that the reagent be made alkaline before the addition of the test solution.

The reagent of Koessler and Hanke gives very little trouble if certain precautions are taken and the writer follows in general their procedure. The continuous use of ice is, however, not always convenient and can be dispensed with if the temperature of the tap water of the laboratory is less than 10°. The sulphanilic acid solution first put into the reagent flask is allowed to come to the temperature of the running water, then the sodium nitrite solution, preferably kept in the ice chest, is added. The mixing of the sulphanilic acid and sodium nitrite is the critical step in the making up of the reagent as diazotisation is a strongly exothermic reaction, so that the solutions should be as cold as possible before mixing. If attention is not paid to this point unsuitable reagents will often result.

When a sodium carbonate solution which has been made up for some time is used as alkali there is an abnormally rapid colour development, a maximum colour of too yellow a tint and of too low an intensity.

The reagent has been used for qualitative purposes in the following manner by the writer: 5 cc. of 1·1 % sodium carbonate are put in a test-tube and 2 cc. of the sulphanilic acid reagent added. The contents are mixed by shaking the tube but without inverting it. If any colour is developed at this stage it is probably due to a dirty test-tube. Within the space of about 1 minute *one drop of urine* is added. One of two distinct phenomena may occur at this stage: (1) *Alkaline diazo reaction, Type A.* In all urines a faint yellow colour is produced which goes on intensifying for the next 3 to 4 minutes. This colour does not generally develop further than a pale orange but may often show predominately a pink tinge and occasionally quite a marked red. (2) *Alkaline diazo reaction, Type B.* In urines from typhoid and measles a bright orange red colour appears in the solution immediately the drop of urine is added, but the colour disappears in a few seconds. (Thereafter there is an intensification of colour due to the substances responsible for Type A reaction.)

1. *Alkaline Diazo Reaction, Type A.*

Some kind of Type A reaction is obtained in all urines. As a rule the more red the colour obtained, the more iminazole is present. Yet it must be emphasised that this method of qualitative examination is a very poor index of the actual amount of iminazole in urine. This is so because of interfering substances in untreated urine. It has previously been shown [Hunter, 1922, 1] that certain purines interfere with colour production, but the presence of uric acid in urine is alone insufficient to account for the great inhibition of colour development which generally prevails. This is clearly seen by a consideration of the 5 urines in Table I. The amount of iminazole present in each was determined by the recent method of Koessler and Hanke [1924], the amounts taken to obtain the readings given below representing 0·05 cc. of original urine. Alongside these are given the approximate readings obtained from 0·05 cc. of untreated urine. The test cylinder in each case is set at 20 mm. and the cylinder containing the histidine colour standard [Koessler and Hanke, 1919] moved to match.

Table I.

Urine No.	Standard cylinder		Test cylinder
	Untreated urine mm.	Prepared urine mm.	mm.
1	4·5	14·5	20
2	6·5	29·5	,,
3	6·5	27·5	,,
4	2·0	4·9	,,
5	1·0	1·7	,,

It will thus be seen that although urines Nos. 2 and 3 show higher readings than Nos. 1, 4 and 5 by both methods, the readings in Nos. 2 and 3 for the untreated urine represent only about $\frac{1}{5}$ of the colour obtainable from the iminazoles actually present in the urines.

On the other hand some urines give a redder colour by this direct method than one would be led to expect from the actual amount of iminazole present. This appears to arise from a decrease in the concentration of the substances inhibiting colour production. Thus the writer [Hunter, 1922, 2] was led to the conclusion that there was an increased excretion of histidine in urine from measles patients through finding a marked diazo reaction of this type, although as shown there were present 310 mg. of histidine monohydrochloride per litre, a not abnormal amount of iminazole as subsequent work has shown. This reaction, it should be noted, was also mistaken for the characteristic reaction given by measles urines before Type B reaction came to the writer's notice. Indeed, there is ample evidence in the literature that this confusion is common. Thus Neubauer and Huppert [1913] quote at least three workers who found a positive Ehrlich's diazo reaction in a fairly large proportion of normal urines. These positive reactions were probably due to the presence of iminazoles and almost certainly not due to the substance which gives the character-

istic test in typhoid and measles. For among several hundreds of normal urines the writer has failed to find one which gives a test of the type given in typhoid or measles.

Of course phenols in urine also contribute to the colour given in Type A reaction. Like the iminazoles the colours for phenols take definite if generally shorter times to develop to a maximum. Likewise purines and tyrosine intensify in colour, though even in pure solution the final colour intensity is not proportional to the amount of the purine or tyrosine present. Ammonium salts and sulphur compounds also give yellow colours, and there are probably several chromogens which either yield a colour in themselves or in some way prevent the diazo reagent from coupling with the iminazoles or phenols.

It may be concluded that the development of a colour predominantly red, of the alkaline reaction A type, is due to the presence of iminazole. The red colour may indicate that the iminazole is present in abnormally large amounts or merely that it is present in normal amounts when the conditions in such a urine are favourable for the coupling of the diazonium salt with the iminazole present. Urocanic acid and histidine are the only two iminazoles so far isolated from urine, though there are probably other iminazoles responsible for Type A reactions.

Type A alkaline diazo reaction is likely to remain of little value as a biochemical test. The interpretation is still too uncertain, as the reagent is too unspecific and too easily interfered with to give reliable information even regarding iminazoles. The normal output of iminazoles in the urine is in the writer's opinion not yet definitely settled although the value of Koessler and Hanke's [1924] contribution in this direction is recognised. But apart from this, practically nothing is yet known of the physiological significance of either a high or a low iminazole excretion. Yet it does appear of importance to distinguish a reaction of Type A from a reaction of Type B such as is obtained in typhoid and measles urines, since Type B reaction holds promise of being a valuable test for certain pathological urines.

2. *Alkaline Diazo Reaction, Type B.*

As far as the writer is aware the alkaline diazo reaction Type B has not hitherto been observed. Its characteristic property is the almost instantaneous appearance of a bright orange red colour of only momentary duration. There is never any doubt as to whether the test is positive or not, as would appear to be in the case of a red foam according to Ehrlich's procedure. Within the past two years the writer has found this type of reaction in the urine of upwards of 30 different measles patients, and in about the same number of typhoid patients. It has been found to persist in some typhoid urines for nearly two weeks, but its onset and disappearance have not been sufficiently studied from a clinical standpoint to permit of discussion here.

A differentiation of two distinct types of diazo reaction in alkaline solution has hitherto been overlooked because of two main factors working together:

(*a*) with the more concentrated reagents of Ehrlich or Pauly the time of colour development is greatly reduced; (*b*) with the relatively large amounts of urine previously used such an excess of the substance responsible for Type B reaction is in the solution that a fading in colour is not readily observable. Further, the colours produced by iminazoles with the old reagents are less stable than those with the Koessler and Hanke reagents. It also appears that the Ehrlich reagent is less sensitive to the substance responsible for Type B reaction than is the new reagent. Thus, a sample of typhoid urine which showed a beautiful, though momentary, orange red colour when dropped into the new qualitative reagent as described above, when tested by the Ehrlich method showed a bright orange colour quickly, but on shaking, the foam was no more red than that obtainable from a urine containing about 0·04 % iminazole.

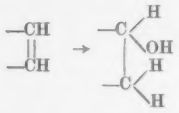
There is little doubt that the substance (or substances) responsible for Type B alkaline diazo reaction is new. From the fact that the diazo reaction has always been recognised as occurring in urines, especially from typhoid and measles, it is also most probably true that this substance was responsible for many of the so-called positive diazo reactions recorded in the older literature. Yet the possibility that iminazoles were responsible for many positive reactions can never be quite eliminated. The method suggested for carrying out the test cannot lead to this confusion.

Within the past two years the writer has made various unsuccessful attempts to isolate the substance from both typhoid and measles urines. The substance disappears in a few days from urine which is allowed to become alkaline, but may be kept for a longer period in acid urines. It is not precipitated by neutral lead acetate but is by mercuric acetate. It disappears on treatment with silver nitrate. It is readily soluble in ethyl alcohol and slowly extracted by butyl alcohol from acidified urine. It reduces alkaline potassium permanganate.

No suggestion of the nature of the substance was obtained until the appearance of a recent paper by Baudisch [1924] on the oxidation of pyrimidines by ferrous bicarbonate. (The only substance previously found by the writer to give a Type B reaction was adrenaline.) Baudisch's work was repeated on uracil. One litre of distilled water, 0·01 g. uracil and 5 g. sodium bicarbonate were boiled for 30 minutes in a 2 l. flask. A small test-tube containing 5 g. ferrous sulphate was then suspended in the neck of the flask. A rubber stopper, through which was a glass tube with a cock on the outside, was then tightly inserted into the neck of the flask with the cock open, and the boiling continued for 10 minutes. The flask was then removed from the flame, the cock closed, and the flask cooled under the tap. The flask was then inverted to mix the ferrous sulphate with the sodium bicarbonate solution. White ferrous bicarbonate was thus precipitated. The cock was then opened to admit air, the stopper removed and the contents of the flask shaken in contact with air until all the iron was changed to the red ferric hydroxide. The fluid was then

filtered and distilled *in vacuo* at 40° to a volume of about 10 cc. One drop of this solution gave with the diazo reagent a bright purplish colour of only momentary duration. The substance responsible for this test according to Baudisch is an unstable oxidation product of uracil, dihydroisobarbituric acid or a condensation product.

The Baudisch oxidation by ferrous bicarbonate is apparently applicable to cyclic compounds containing an ethylene group. This group is common to the pyrimidines and is oxidised by ferrous bicarbonate by the addition of one molecule of water as follows:



Dihydroisobarbituric acid is unstable and condenses to a yellow-coloured pigment, a bimolecular pinacone-like compound, of an auto-oxidisable nature. The evaporated fluid (dihydroisobarbituric acid or the yellow pigment) reduces ammoniacal silver nitrate solution, gives a blue colour with phosphomolybdic acid and reduces methylene blue.

The writer obtained a reduction with ammoniacal silver nitrate, a blue colour with phosphomolybdic acid and a marked diazo reaction of Type B. The colour obtained with this oxidation product of uracil was however much more purple than that obtained from typhoid urines.

Indole contains an ethylene group like uracil and this was tested as described above for uracil. The final solution gave a reaction of the same type—a momentary orange colour which quickly faded to yellow. This colour was much more like that obtained from the typhoid urines but less bright in colour.

The iminazoles contain an ethylene group; histidine, therefore, was treated in the same manner. The final solution from histidine gave a reaction still of the same type, but the colour was much more yellow than that from typhoid urine. It may be noted here that after treatment with ferrous bicarbonate the solution showed no signs of a diazo reaction of Type A, a finding which shows that the iminazole ring suffers some change.

It cannot be concluded from these findings that the substance in typhoid urine is an oxidation product of indole, merely on the ground that the colour given by oxidised indole is nearer that from the typhoid urine than the other substances tested; for the substance in typhoid urine has not yet been obtained in sufficiently pure solutions to warrant the conclusion that an orange red is its characteristic colour, and, further, there is a wide variety of substances in the body presumably capable of the ferrous bicarbonate oxidation.

It is remarkable that the oxidation products of cytosine, uracil, indole and histidine all give a diazo reaction of Type B. This constancy of finding would suggest that Type B diazo reaction indicates some common atomic con-

figuration such as has been suggested by Baudisch for dihydroisobarbituric acid or the yellow pigment. (Whether it is dihydroisobarbituric acid or its condensation product which is responsible for the reaction does not appear quite clear from Baudisch's paper.) The substance responsible for Type B reaction in typhoid urines has thus probably a part of its molecule of the same configuration as the oxidation products of uracil or of other cyclic compounds with an ethylene linkage. The substance in typhoid urine reduces ammoniacal silver nitrate solution as does the oxidation product of uracil. Both substances are unstable in alkaline solution and reduce alkaline potassium permanganate.

M. Weiss's [1920] conclusion that the substance responsible for the diazo reaction in urine is urochromogen is supported by Baudisch's finding that the oxidation product of uracil condenses to a yellow pigment. It would however seem to be necessary to have a reliable analysis of pure urochrome and also an analysis of the substance in question before the hypothesis of Weiss can be regarded as an established fact.

Further details of the properties of the oxidation products of uracil, or of substances capable of this ferrous bicarbonate oxidation, may suggest new methods for the isolation of this substance from urine. If the substance does not actually prove of importance from a clinical point of view it is at least interesting as a pathological finding and may in time prove to be an exaggeration of some normal physiological process.

Conclusion.

An attempt has been made to systematise the different types of diazo reaction in normal and pathological urines. The Ehrlich diazo reaction in acid solution now used extensively to distinguish different types of jaundice, is only referred to as there has never been any question of its specificity in biological fluids for bilirubin. The diazo reaction in alkaline solution, or more specifically in sodium carbonate solution, has been divided into two types called for convenience Type A and Type B. Type A reaction is characterised by a gradual development of colour until a relatively stable maximum is reached. Type B reaction is characterised by an immediate development and a very rapid disappearance of the colour.

All urines give some form of Type A reaction. The substances in urine mainly responsible for it are iminazoles, phenols, purines and probably unknown chromogens. Type B reaction is given only by pathological urine, especially that from typhoid and measles. The substance responsible for Type B reaction is most probably a hitherto unisolated substance. Some indications of its probable nature have been suggested.

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V. CARNOSINE OF MUSCLE AND IMINAZOLE EXCRETION IN THE URINE.

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One of the conclusions in a recent communication by the writer was that the carnosine content of striated muscle in the cat fluctuates [Hunter, 1924]. It was shown that the carnosine content of white muscle could be lowered; it is now indicated that it can be lowered or raised. Lack of exercise was suggested as a possible cause of the fall in carnosine values shown to occur in certain caged animals. Further work has shown that diet has a definite influence on the carnosine content of muscle, although it has not been disproved that exercise plays a part.

It was previously indicated that a rapid fall in the carnosine content of muscle might be accompanied by an increased iminazole excretion in the urine. This hypothesis appeared true when it was found that two cats, with a high carnosine value in the muscle of an amputated leg, passed, during the following 24 hours—when presumably the muscles were rapidly losing carnosine—urines which showed a more than usually red colour with the diazo reagents.

On the publication of a method for the estimation of iminazoles in urine by Koessler and Hanke [1924] it was decided to test this hypothesis further. The results reported here are evidence in its favour.

The estimation of iminazoles in the urine is of recent date, the first attempt perhaps being made by the writer in 1922 [Hunter, 1922, 1]. It was then shown that a litre of a mixed sample of urine from patients suffering from measles contained 310 mg. iminazole calculated as histidine monohydrochloride, and approximately 100 mg. of the pure substance were isolated and identified.

The recent method of Koessler and Hanke is suitable for the routine estimation of iminazoles in urine. The use by these workers of sodium hydroxide along with lead acetate is more satisfactory than that of baryta as used by the writer. The subsequent evaporation of the filtrate is also indispensable.

Experimental.

1. *Changes in the Carnosine Content of Muscle.*

In Table I is shown the carnosine content of the muscles, gastrocnemius, plantaris and soleus, in the amputated limbs of five cats, and after a varying interval the carnosine content of the corresponding muscles of the opposite limbs. The first date in each case is the day on which the right leg was amputated and the carnosine content of the muscles determined; the second date, the day on which the cat was killed and the carnosine values of the corresponding muscles of the left leg estimated.

Table I.

			Gastrocnemius		Plantaris		Soleus	
Cat No.	Date	Wt. of cat g.	Wt. of muscle g.	% carnosine	Wt. of muscle g.	% carnosine	Wt. of muscle g.	% carnosine
13	26. ii. 24	2681	17·870	0·356	5·184	0·298	3·030	0·051
	2. iv. 24	2710	17·790	0·431	4·859	0·288	3·270	0·050
15	26. iii. 24	1915	13·055	0·131	4·349	0·097	1·710	0·065
	7. iv. 24	1740	11·975	0·023	—	—	1·450	0·064
18	11. vi. 24	2100	13·370	0·014	4·230	0·014	2·212	0·087
	20. vii. 24	3170	22·770	0·258	7·030	0·214	3·500	0·052
			Lateral gastrocnemius					
19	2. vi. 24	3170	9·480	0·386	6·140	0·389	2·890	0·057
	5. vi. 24	2810	8·170	0·166	5·520	0·163	2·900	0·051
			Lateral gastrocnemius					
20	31. v. 24	3168	11·195	0·286	7·519	0·262	3·507	0·082
	12. vi. 24	2750	8·070	0·016	4·534	0·022	3·315	0·084

Cat No. 13 belonged to the same litter as cats Nos. 5 and 6 of a previous paper [Hunter, 1924]. Cats Nos. 5 and 6 gave very low values for the gastrocnemius, viz. 0·01 % and 0·007 % and cat No. 13 would probably have given like values had it been killed at the same time. It was however fed on meat until it was in a moderately good condition. When the right leg was amputated the gastrocnemius contained 0·356 % carnosine. The weight of the cat after the amputation was 2681 g. It recovered well although it lost weight for the following two weeks. A good meat diet was given and the weight increased progressively, reaching 2710 g. when the animal was killed, about 5 weeks after the operation. The weights of the two gastrocnemii were practically the same, but the second carnosine value was 0·075 % greater than the first. This increased carnosine value, however, is not borne out in the values from the plantaris. The soleus values remained, as usual, constant. Owing to the cat's loss of weight during the two weeks following the amputation the carnosine content of the gastrocnemius probably fell during that period.

That the carnosine content of muscle may be increased is clearly shown in the case of cat No. 18. This animal was starved for 15 days during which time

its weight fell from 3000 to 2100 g. The carnosine content of the gastrocnemius at the end of the starvation was 0·014 %. For the next three weeks the cat was kept on a maintenance diet of meat and during the following three weeks was given a larger amount so that the weight increased to about 1000 g. more than at the date of amputation. (Further data on this cat are given in Table II.) The carnosine content of the gastrocnemius was now 0·258 %, of the plantaris 0·214 %, and of the soleus 0·052 %. The change in the value from the soleus is unusual.

Cats Nos. 15, 19 and 20 were starved after the operation. In all cases the carnosine values of the white muscles fell.

It may be concluded from the results in Table I that the carnosine content of striated muscle falls during starvation and increases after a meat diet if the weight of the cat increases, even when cats are caged. It is thus clear that if exercise influences the carnosine content of muscle it is not a controlling factor.

2. *Urinary Iminazole of Cats.*

When cats are on a constant diet their daily urine output is fairly regular; and, since they show probably a wider variation in the carnosine content of their muscles than any other laboratory animal, they are peculiarly suitable to use in order to determine whether there is an increased iminazole output in the urine when the carnosine content of the muscle falls rapidly. The iminazole in the urine was estimated according to a modified Koessler and Hanke [1924] procedure. (This modification it is hoped to deal with in detail in a forthcoming paper.) The total daily output of urine was used for each estimation with proportionate amounts of lead acetate and sodium hydroxide. The values are given as "iminazole" according to the suggestion of Koessler and Hanke.

The five cats reported in Table II were all in excellent condition when the urine examination was begun and the carnosine content of their muscles was probably high.

It is notable that with the exception of cat No. 19 the urine on the first day of starvation showed the highest iminazole content over the periods recorded. (Cat No. 21 may be regarded as starving, as it would not at first eat the bread after a diet of meat.) There tends also to be an increased iminazole excretion following amputation, especially so in cats Nos. 19 and 20, where the carnosine value in the muscle is shown to be high. This is less marked in cat No. 18, where the value in the amputated limb was low. The value of 6·0 mg. in this cat following amputation represents two days' output of urine; in such cases the value is generally about double, even if the volume is little changed. This feature is again salient in the same cat on June 14th and 27th, and in cat No. 21 on June 23rd.

The urine from cat No. 17 was not examined over a sufficiently long period to provide conclusive evidence of a fall in iminazole output, as the cat unex-

Table II.

Cat no. 17.

Date	Urine cc.	Iminazole mg.		Diet and remarks
May 29	64	12·5	↑ Starvation	Wt. of cat 3520 g.
„ 30	none	—		
„ 31	41	7·8		
June 1	none	—		
„ 2	38	6·8		
„ 3	none	—		
„ 4	47	7·5		1 hour under chloroform
„ 5, 6	none	—		
„ 7	47	6·5		
„ 8	65	9·7	↓	

Cat died on June 9th. Wt., June 9th, 3050 g.
Carnosine: r. plantaris 0·181 %; l. plantaris 0·184 %.

Cat no. 18.

Date	Urine cc.	Iminazole mg.		Diet and remarks
May 28	105	12·5	↑ Starvation	Wt. 3000 g.
„ 29	none	—		
„ 30	73	8·2		
„ 31	none	—		
June 1	83	9·4		
„ 2	none	—		
„ 3	57	6·0		
„ 4	35	3·5		
„ 5	49	4·2		
„ 6	none	—		Given 200 mg. carnosine intravenously
„ 7	45	21·5		
„ 8	33	3·2		
„ 9	25	1·2		
„ 10	45	1·3		
„ 11	none	—	↓	Wt. of cat 2100 g.; carnosine 0·014 % (gastrocnemius)
„ 12	78	6·0	↑ 90 g. meat per day	
„ 13	none	—		
„ 14	78	10·8		
„ 15	38	8·0		
„ 16	52	7·6		
„ 17	42	4·6		
„ 18	45	4·4		
„ 19	43	4·8		
„ 20	30	4·0		
„ 21	42	4·5		
„ 22	41	5·3		
„ 23	40	5·2		
„ 24	36	4·3		
„ 25	40	3·9		
„ 26	none	—		
„ 27	55	8·5		
„ 28	41	6·1		
„ 29	42	7·0		
„ 30	39	6·1		
July 1	lost	lost		Wt. of cat 2150 g. Given 500 mg. carnosine orally
„ 2	40	7·2	↓	

Cat fed from July 2nd till killed, July 20th, on 200 g. of meat per day.
Wt., July 20th, 3170 g.; carnosine 0·258 % (gastrocnemius).

Cat no. 19.

Date	Urine cc.	Iminazole mg.		Diet and remarks
May 27	35	7·9	Starvation	Wt. 3680 g.
„ 28–30	none	—		
„ 31	35	9·0		
June 1–2	none	—		June 2nd, carnosine 0·386 % (lat. gast.)
„ 3	35	14·0		
„ 4	16	4·5		

Cat died June 5th. Wt., June 5th, 2810 g.; carnosine 0·166 %. (lat. gast.)

Cat no. 20.

Date	Urine cc.	Iminazole mg.		Diet and remarks
May 31	108	24·2	Starvation	
June 1	none	—		Wt. of cat 3670 g.; carnosine 0·286 % (lat. gast.)
„ 2	140	22·0		
„ 3	none	—		
„ 4	50	7·2		
„ 5	66	6·3		
„ 6	40	3·0		
„ 7	35	3·2		
„ 8	32	3·0		
„ 9	none	—		
„ 10	32	2·2		
„ 11	45	4·5		

Cat killed June 12th. Wt., June 12th, 2750 g.; carnosine 0·016 %. (lat. gast.)

Cat no. 21.

Date	Urine cc.	Iminazole mg.		Diet and remarks
June 14	84	12·7	Bread and water and little milk	
„ 15	157	4·0		Some faeces in urine
„ 16	60	8·0		
„ 17	132	6·5		
„ 18	92	4·2		
„ 19	121	4·5		
„ 20	175	4·8		„ „ „ „
„ 21	175	3·0		„ „ „ „
„ 22	none	—		
„ 23	105	7·8		
„ 24	170	7·0		
„ 25	168	4·2		
„ 26	214	5·7		„ „ „ „
„ 27	150	2·5		
„ 28	80	2·4		
„ 29	181	4·0		
„ 30	98	5·5		
July 1	163	2·5		
„ 2	178	3·6		

Cat kept on above diet till Aug. 4th, when killed. Wt., Aug. 4th, 1860 g.; carnosine 0·023 %.

pectedly died after 10 days' starvation, showing a loss of about 500 g. in weight. At death the carnosine content of the corresponding plantaris muscles was 0·181 and 0·184 %. Even taking account of the irregularity of the urine output the values are relatively high and would probably have fallen after the remaining carnosine had left the muscle.

The iminazole values for cat No. 18 show a fairly progressive fall during the starvation period. For a few days following amputation there is a slight rise in values, after which, for a period of nine days, there is a very regular urine output and the iminazole values show little variation. During the last week of the urine examination the iminazole output again rises owing to an increase in the muscle carnosine following a meat diet.

Cat No. 19 serves to demonstrate a high iminazole output immediately following amputation. This cat also died for unknown reasons after the loss of about 800 g. in weight, the gastrocnemius still showing 0·166 % carnosine. The values as in cat No. 17 are higher than usual throughout.

Cat No. 20 shows a progressive fall in the iminazole of the urine as starvation continues. Over a period of 11 days the carnosine content of the gastrocnemius falls from 0·286 to 0·016 %.

Cat No. 21 was maintained on a diet of bread and water with a little milk added. The urinary output is very high and relatively constant and the iminazole values show some fluctuation. During the first 10 days, however, the values are as a whole higher than in the succeeding period.

During the starvation period, on June 6th, cat No. 18 was given 200 mg. carnosine intravenously. The urine on the following day showed a decided rise in iminazole, representing about one-tenth of the administered carnosine. On July 1st, when on a meat diet, this same cat was given 500 mg. of carnosine sprinkled over the surface of the meat which the cat ate under observation. The urine on the following day showed no marked rise in iminazole output. It is thus clear that a cat can ingest relatively large amounts of carnosine without increase in iminazole output. It is less clear how the cat deals with iminazole actually in the blood stream. For the carnosine was given intravenously at a single injection and at least part of the 21·5 mg. recorded was carnosine as shown by a decrease in colour value on hydrolysis and an increased Knoop test [Hunter, 1922, 2]. However 200 mg. of carnosine is probably not an abnormal amount to be thrown into the circulation on a single day in a cat whose muscle is rapidly losing carnosine—assuming that the carnosine lost by muscle enters the blood stream. For as shown in cat No. 19, the carnosine content of the gastrocnemius fell to the extent of 0·22 % in three days, representing a little over 0·7 g. per kg. of muscle per day. Taking the muscle mass of cat No. 19 as 1400 g. this indicates a loss from the total muscle of rather more than 0·9 g. carnosine. Allowing for red muscle and other muscle with less carnosine than the gastrocnemius, it seems not unlikely that at least 500 mg. may be dealt with by the blood stream per day from this source alone.

The balance of evidence from the urines of the five cats represented in Table II dips in favour of the view that some of the urinary iminazole comes from the carnosine of muscle. Yet there are irregularities in the iminazole excretion which cannot be accounted for on this assumption. Perhaps a clear proof is not to be expected, for, if carnosine is a reserve material, it is probably in large part utilised by the starving animal. Further, when carnosine rapidly

disappears from muscle only a fraction may reach the blood stream, and of this fraction only a part of the carnosine or its breakdown products is likely to reach the urine with the iminazole ring intact. An increase in the iminazole content of the blood will require to be demonstrated before it can be determined whether the carnosine lost from muscle enters the blood stream or is directly utilised by muscle. There is not yet a suitable method for the estimation of iminazoles in small quantities of blood. The writer has found by a fractional precipitation that there are at least 1 to 2 mg. of carnosine per 100 cc. of ox blood.

An attempt was made in several of the cat urines to determine the main iminazole present. In one cat the lead acetate filtrates were collected and precipitated by mercuric acetate. Nearly all of the colour giving substance was precipitated in this manner. The substance was also precipitated in a *slightly acid solution* by silver nitrate. The final solution obtained after several precipitations in this manner gave a colour which developed more quickly and was finally more red than that obtained from histidine. The solution of the substance was strongly acid to litmus and rapidly decolorised alkaline permanganate. It gave a strongly positive Knoop test.

Its behaviour in the silver precipitation, its acid reaction, and its unsaturation parallel the properties of urocanic acid. It is not stated in the literature whether urocanic acid gives a positive Knoop test and its colour with the modern diazo reagents has not been compared with that obtained from histidine.

It was estimated on an arbitrary molecular colour value of 130 million mm. that there was about 0·2 g. of the substance in the solution above referred to, yet crystals could not be obtained directly in the solution, nor with picric or nitric acids. It might be supposed from the literature on urocanic acid that at least a picrate should have been obtained under the conditions were the substance urocanic acid[1].

[1] Through the courtesy of Professor Andrew Hunter of this University the writer has obtained some pure urocanic acid. A saturated solution of this substance (about 0·2 % at 20°) is quite negative to Knoop's test. Its behaviour with the diazo reagents is also noteworthy. A solution containing 0·04 mg. per cc. of $C_6H_6O_2N_2 2H_2O$, when tested for histidine and carnosine, as described in the paper which follows, gives a molecular colour value of approximately 109 million mm. The final colour developed by urocanic acid with the diazo reagents is, however, more yellow than that produced by histidine and its time of colour development is much more prolonged, viz. 12–14 mins. The intensity of colour produced is proportional to the amount of urocanic acid present.

Although the substance from cat's urine above described is acid in reaction, unsaturated and gives a colour like iminazoles with the diazo reagents, it is most probably not urocanic acid because it is much more soluble in water, it gives a Knoop test and behaves differently with the diazo reagents as regards time of colour development and shade of colour.

SUMMARY.

1. The carnosine content of striated muscle in the cat can be lowered by starvation, and if lowered can again be raised by a meat diet.

2. When carnosine disappears rapidly from muscle there is evidence of an increased excretion of iminazole in the urine.

Acknowledgment is due to the Medical Research Committee, University of Toronto, for the animals used.

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VI. COLOUR STANDARDS FOR USE IN THE DETERMINATION OF IMINAZOLES.

By GEORGE HUNTER.

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(*Received December 8th, 1924.*)

The use of a mixture of Congo red and methyl-orange solutions was recommended by Koessler and Hanke [1919] when these workers published their method for the determination of iminazoles. Suitable mixtures of the two pigments gave colours which matched closely the colour developed by mixing solutions of histidine or other iminazole with the diazo reagent in a sodium carbonate solution.

It is a good rule in colorimetric work to use a standard made from the substance to be measured rather than to employ an artificial standard; but in the case of iminazoles the use of an artificial standard is almost indispensable, chiefly because it is impracticable to make up the standard and test solutions exactly at the same time, a procedure which the relative instability of the colours obtained would seem to demand. Further, iminazoles are as a rule difficult to prepare, and with the exception of histidine are not on the market. A reliable artificial standard will thus always serve as some check on the purity of iminazole solutions of different workers.

The stock solutions of Congo red and methyl-orange recommended by Koessler and Hanke [1919] were stated to keep indefinitely. This seems to be true. Recently, however, mainly to test this point, the writer had occasion to revise his standards. Previously, vacuum dried Grübler's Congo red and methyl-orange had been used as recommended originally by Koessler and Hanke. In this instance it was thought desirable to recrystallise the Grübler products and compare the standard made from these with the old standard. The new standard was found to have about 25 % greater colour value than the old standard. This finding was not, as at first suspected, due to a diminution of colour in the old stock solutions through standing, but was due to impurities in the Grübler's Congo red and methyl-orange.

The Congo red of four different manufacturers was tested and ranged from 40 % to 75 % of the colour value of the purified Congo red. Different methyl-orange products showed a like range of variation.

This difference in colour value is borne out by the residues obtained on ignition from the purified and commercial products. Both Congo red and methyl-orange are sodium salts, and thus leave a residue on ignition. The theoretical amount of sodium, weighed as sodium sulphate, can be calculated from the formulae. Thus Congo red, $C_{32}H_{22}O_6N_6S_2Na_2$, contains 6·62 % of sodium or 20·44 % as sodium sulphate. The formula of methyl-orange is $C_{14}H_{14}O_3N_3SNa$, which yields 21·71 % sodium sulphate.

Before dealing with the ash values the method of purification of the pigments will first be given.

1. *Congo red.* About 10 g. of Grübler's Congo red with 150 cc. of water are heated in a beaker placed in a boiling water-bath and stirred until the sediment is at a minimum, when the contents of the beaker are filtered through a Buchner previously heated by steam. The filtrate is reheated on the water-bath and 150 cc. of 95 % alcohol added. It is then placed in an ice chest over night. The crystal mass is filtered by suction, washed with alcohol, and allowed to dry in the open. The yield (about 7 g.) is ground in a mortar, redissolved in 100 cc. of hot water, filtered and reprecipitated by an equal volume of alcohol. The final air dry product is ground and heated in a water oven until constant in weight, when it is assumed to be water free: Yield 4–5 g.

2. *Methyl-orange.* A beaker containing 200 cc. water and 15 g. of Schuchardt's methyl-orange, which was found to give a rather higher colour value than Grübler's, is placed in a boiling water-bath. The solution is filtered through a hot Buchner, and the filtrate allowed to cool, when the methyl-orange immediately separates out. The solution is allowed to stand at 0° overnight. The methyl-orange is then filtered off, washed with cold water, and dried in the open. About 10 g. of dry material are then redissolved in 100 cc. of water and the procedure, as described, repeated. The final air dry product is ground and brought to constant weight in the water oven. A final yield of almost 8·0 g. is obtained.

It is advisable to keep the substance in an evacuated calcium chloride desiccator rather than in one containing sulphuric acid, as Congo red will darken in colour in presence of the acid.

The residue on ignition as sodium sulphate is perhaps the most convenient index of the degree of purity of Congo red or of methyl-orange. The only disadvantage of ashing is that a little of the sodium appears to volatilise on combustion of the carbon before the addition of the sulphuric acid.

The following percentages of sodium sulphate were found in Grübler's products and in the Congo red and methyl-orange twice recrystallised. The high residue on ignition from Grübler's Congo red is due mainly to sodium sulphate and from the methyl-orange to sodium chloride.

	Congo red % Na_2SO_4	Methyl-orange % Na_2SO_4
Grübler's	41·95	49·40
Twice recrystallised	19·42	20·45
Theoretical	20·44	21·71

The above findings necessitate a new basis for the making of the colour standards used in the estimation of iminazoles. If the quantities of stock Congo red and methyl-orange recommended by Koessler and Hanke are taken from pure solutions of these pigments the molecular colour values of the different iminazoles are materially lowered and a correction of all the work previously done is required. This, however, is unnecessary if a new colour standard is made to give a molecular colour value of 114 million mm. for histidine—the value originally given by Koessler and Hanke. The writer [Hunter, 1921] previously found a rather higher molecular colour value for histidine so that his colour standard was probably more dilute than that used by Koessler and Hanke. For this reason the molecular colour value of carnosine is now appreciably lowered. This does not affect the writer's results previously published for carnosine as the molecular colour value was accurate according to the colour standard then used.

The stock solutions are made as follows:

Stock methyl-orange. This consists of 0·1 g. of pure methyl-orange made to 100 cc. with water.

Stock Congo red. This consists of 0·2 g. of pure Congo red made to 100 cc. with water.

Koessler and Hanke originally recommended 0·5 % Congo red, but with pure Congo red this is too concentrated for accurate pipetting. These authors also used 10 cc. of alcohol per 100 cc. This appears quite unnecessary as Congo red is more soluble in water than in alcohol. Alcohol, indeed, gives the solution a turbid appearance for at least some days after mixing.

Histidine Colour Standard (*S*). About 80 cc. of distilled water are taken in a 100 cc. flask and 0·40 cc. stock Congo red added. After mixing, 0·06 cc. stock methyl-orange is added and the flask filled to the mark with water.

This colour standard, which contains a rather greater proportion of Congo red than that originally recommended by Koessler and Hanke [1919], matches excellently the colour produced by histidine and at the same time keeps the molecular colour value of this substance at about 114 million mm.

The colour produced by carnosine is slightly redder than that obtained from histidine but may be easily read against the above histidine colour standard. For this reason the writer has discontinued the use of a separate colour standard of carnosine.

The molecular colour values of histidine and carnosine were determined as follows: aqueous solutions were made containing 0·03 mg. histidine, 0·05 mg. histidine monohydrochloride, 0·03 mg. carnosine and 0·05 mg. carnosine nitrate per cc. respectively.

From each of the four test solutions thus prepared five readings were obtained using amounts differing by 0·2 cc. The reading given by 0·01 mg. of the substance is thus obtained and the molecular colour values calculated as shown below. In each case the molecular weight $\times$ the reading for 0·01 mg. $\times$ 10^5 = the molecular colour value in mm.

1. *Solution containing* 0·03 *mg. histidine per cc.*

Amount of test solution used cc.	Colour standard reading, *S* mm.	Test solution reading, *T* mm.
1·0	22·1	20
0·8	17·5	,,
0·6	13·2	,,
0·4	8·7	,,
0·2	4·4	,,

From these readings 0·01 mg. histidine gives a reading of approximately 7·4 mm.; whence the molecular colour value of histidine is

$$155 \times 7{\cdot}4 \times 10^5 = 114{\cdot}7 \text{ million mm.}$$

2. *Solution containing* 0·05 *mg. histidine monohydrochloride per cc.*

Amount of test solution used cc.	Colour standard reading, *S* mm.	Test solution reading, *T* mm.
1·0	26·8	20
0·8	21·5	,,
0·6	16·2	,,
0·4	10·8	,,
0·2	5·4	,,

From these readings 0·01 mg. histidine monohydrochloride gives a reading of approximately 5·4 mm.; whence the molecular colour value of histidine is

$$209{\cdot}5 \times 5{\cdot}4 \times 10^5 = 113{\cdot}1 \text{ million mm.}$$

3. *Solution containing* 0·03 *mg. carnosine per cc.*

Amount of test solution used cc.	Colour standard reading, *S* mm.	Test solution reading, *T* mm.
1·0	18·6	20
0·8	15·0	,,
0·6	11·1	,,
0·4	7·4	,,
0·2	3·8	,,

From these readings 0·01 mg. carnosine gives a reading of approximately 6·2 mm.; whence the molecular colour value of carnosine is

$$226 \times 6{\cdot}2 \times 10^5 = 140{\cdot}1 \text{ million mm.}$$

4. *Solution containing* 0·05 *mg. carnosine nitrate per cc.*

Amount of test solution used cc.	Colour standard reading, *S* mm.	Test solution reading, *T* mm.
1·0	24·8	20
0·8	19·5	,,
0·6	14·6	,,
0·4	9·8	,,
0·2	4·9	,,

From these readings 0·01 mg. carnosine nitrate gives a reading of approximately 4·9 mm.; whence the molecular colour value of carnosine is

$$289 \times 4{\cdot}9 \times 10^5 = 141{\cdot}6 \text{ million mm.}$$

The molecular colour value of histidine as determined from the free base and from the monohydrochloride differs by about 1 %. It may be taken at 114 million mm. as measured by the new standard.

With the same colour standard the molecular colour value of carnosine as determined from the free base and from the nitrate is 141 million mm.

SUMMARY.

It has been shown that the best commercial Congo red and methyl-orange are not suitable, without purification, for use as artificial standards in colorimetric work.

A colour standard made from pure Congo red and methyl-orange suitable for the estimation of histidine or carnosine is described.

The purity of other dyes used for colorimetric standards should be ascertained before standardising against particular iminazoles, phenols or other substances measurable by the diazo method.

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VII. THE PRESENCE OF VITAMIN A IN YEAST FAT.

By ETHEL MARJORIE LUCE and IDA SMEDLEY MACLEAN.

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(*Received December 12th, 1924.*)

It is well known that yeast is comparatively rich in vitamin B and is frequently used as a source of this substance. The evidence as to the existence of the fat-soluble A vitamin in yeast is however not so clear and the presence of this accessory factor in yeast does not seem to be generally recognised.

Hopkins [1912], in his classic paper on the importance of accessory factors in normal dietaries, described experiments which certainly indicate the presence of the A vitamin in yeast. In these experiments rats were fed on a basal diet consisting of casein, starch, sugar, lard and salts; the addition of 4 % of milk to this dietary induced normal and continued growth. Protein-free alcoholic extracts of milk-solids and of yeast in very small amounts produced a similar acceleration of growth; it is also stated in the same paper that a minute amount of the ether-soluble fraction of the total alcohol extract of yeast induced great acceleration of growth. The significance of this observation seems to have escaped general notice. The diet used was deficient in the three accessory factors A, B and C, and the addition of yeast to the diet would certainly furnish an adequate supply of the B vitamin. It is improbable however that the ether-soluble fraction of the alcohol extract of yeast contained any significant amount of the B vitamin since as a general rule the B vitamin is not extracted by ether [Medical Research Committee, 1924]. It seems almost certain therefore that the acceleration of growth obtained in these experiments was caused by the addition of the ether-soluble A vitamin. Macallum [1919] also found that the addition of 2 or 6 % of dried brewer's yeast to a diet deficient in vitamins A and B induced satisfactory growth in rats for short periods of time.

It seemed to us desirable to investigate directly whether acceleration of growth in rats is produced by the addition of yeast fat (or more accurately, of the ether-soluble constituents of yeast) to a diet which already contained a supply of the B vitamin. In this case if the yeast fat was effective in inducing growth, the presence of the A vitamin in yeast would be satisfactorily confirmed.

A study of yeast fat by one of us [Smedley MacLean, 1922] has shown that the percentage of fat in yeast may be considerably increased by incubating the yeast in a solution of sugar, such as fructose, glucose or sucrose. It is

difficult however to obtain the increased amount of fat by directly extracting the dried yeast with ether; on the other hand, it is quite easily extracted if the yeast is first boiled for two hours with a normal solution of hydrochloric acid. The carbohydrate of the yeast is hydrolysed and both carbohydrate and protein pass into the acid solution, the fat being readily extracted from the dried residue. A comparison of the A vitamin content of the yeast fat obtained by direct extraction with that of the fat obtained from the yeast residue after hydrolysis of the carbohydrate was undertaken. It seemed of interest to determine whether the conditions which led to an increase of fat in the yeast cell also led to an increase of the A vitamin.

Almost all the additional fat formed when the yeast is incubated in a sugar solution is contained in the fat extracted after treatment of the yeast with the normal acid solution; it was therefore interesting to determine whether such a sample of fat contained the A vitamin or whether this vitamin was contained almost entirely in the first direct ether extract.

The experiments were carried out according to the method recommended by Zilva and Miura [1921] and the basal diet was that used by these observers containing the B and C vitamins but free from A vitamin. It consisted of a mixture of casein 20 %, starch 50 %, salts 5 %, hardened cottonseed oil 15 %, marmite 5 %, and decitrated lemon juice 5 %. After the usual preliminary period on the basal purified diet, indicated by dotted lines in the growth curves (Figs. 1, 2 and 3), the various substances to be tested were given to the animals daily for 28 days. The calcium content of the bones of both hind legs (femur, tibia and fibula) was estimated in each case by Aron and Sebauer's method [1910].

The results of these experiments are shown in Figs. 1, 2 and 3. The rats in each group are of the same sex and taken from the same family.

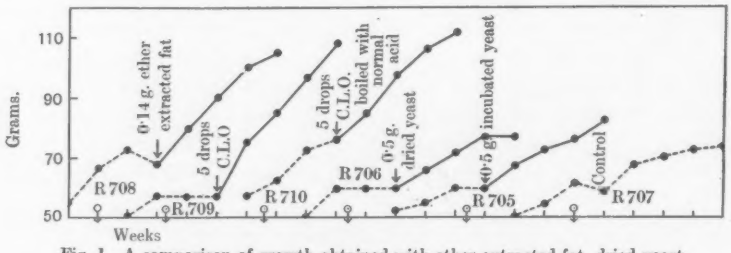

Fig. 1. A comparison of growth obtained with ether extracted fat, dried yeast, incubated yeast, and maximal doses of cod-liver oil.

	% Ca in dry weight of bones		
R 707	16·2	R 710	19·4
R 705	16·1	R 709	19·1
R 706	15·8	R 708	18·1

In Fig. 1 comparison is shown of the growth obtained with daily doses (*a*) of fat obtained by directly extracting dried yeast with ether (0·14 g.); (*b*) of dried yeast (0·5 g.); (*c*) of yeast incubated in carbohydrate solution to

increase the fat content and then dried (0·5 g.); and (*d*) of cod-liver oil (5 drops daily, about 0·14 g.). The results show absence of growth obtained with both specimens of dried yeast, positive growth with the yeast fat and with the cod-liver oil.

In Fig. 2 the results are given of the tests with the fat obtained by direct extraction of the dried yeast with ether and with the fat obtained from the residue by boiling it with acid and then drying it and extracting it with ether. In the first case there was growth, in the latter not.

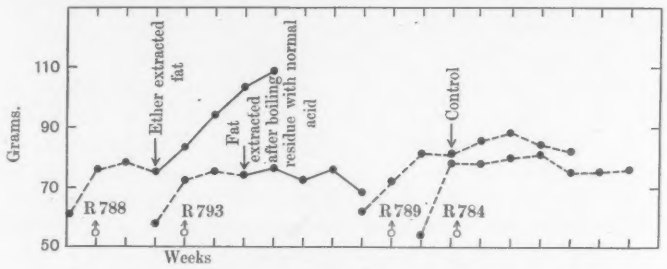

Fig. 2. A comparison of growth obtained with ether-extracted and hydrolysed fat from the same yeast.

% Ca in dry weight of bones

R 784	12·6	R 793	14·7
R 789	14·2	R 788	16·3

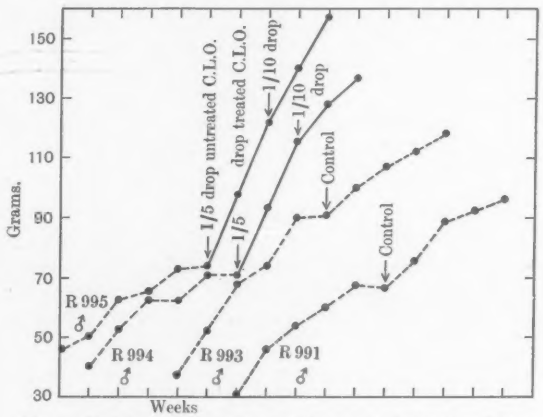

Fig. 3. Test for the destruction of the growth factor in cod-liver oil by boiling with normal acid (minimal dose).

R 991	17·8	R 994	18·7
R 993	16·1	R 995	21·9

As far as these experiments go they indicate that the A vitamin is readily extracted by ether with the more easily obtained fat and there is no evidence that the residual fat is associated with the A vitamin.

Tests were then made to determine how far the process of hydrolysis used had a destructive effect on the A vitamin. A sample of cod-liver oil was submitted to the same process of boiling with normal acid and the effect of the oil thus treated compared with that of the original sample. If a dose of 5 drops of each of the two samples was given daily no difference could be detected in their results (Fig. 1), but with a minimal dose the effect becomes apparent. Fig. 3 shows that with a minimal dose of $\frac{1}{10}$ of a drop of oil there was some destruction of the growth factor in the sample that had been heated with the acid. The growth of rat 994 ran parallel with that of 995 until the dose was reduced from $\frac{1}{5}$ to $\frac{1}{10}$ of a drop.

Discussion of Results.

The negative results obtained when dried yeast was added to the basal diet (Fig. 1) are probably explained by the dosage. Dried brewer's yeast used in these experiments contains about 3 % fat and 0·5 g. dried yeast contains therefore only about 0·015 g. of fat, a quantity insufficient to maintain growth; the dried yeast after incubation contains three or four times as much fat as the original sample but we have been unable to obtain evidence that there has been any corresponding increase in the growth factor. It was difficult to feed large doses of the dried yeast and the dosage is more satisfactorily tested by giving the ether-soluble constituents; the results obtained when a dose of 0·15 g. of the yeast fat was administered daily (Fig. 1) show that the growth factor is present in yeast; an increased calcium content is also found on analysis of the bones.

The striking difference in the results obtained when (*a*) the fat directly extracted by ether and (*b*) the fat dissolved by ether after the residue has been treated with acid shows that the A vitamin probably occurs in the cell in an easily extractable form and is not associated with the residual fat which is more firmly retained in the cell; we did not obtain any indication that increase of vitamin accompanied the increase of fat which occurs when the yeast is incubated in a sugar solution.

The yeast used in these experiments was ordinary brewery yeast grown in the absence of direct sunlight, the surface of the wort only being exposed to diffuse light. The question arises as to whether the yeast was able to synthesise the fat-soluble vitamin in the absence of any direct solar radiation or whether this accessory factor was picked up by the yeast from the medium and concentrated in the yeast.

Since the wort in which the yeast is grown consists essentially of an aqueous extract of malted barley with or without the addition of carbohydrate, if the fat-soluble vitamin has not been synthesised by the yeast it must have been preformed in the malted barley.

Steenbock, Kent and Gross [1918] have shown that whereas barley is rich in the water-soluble B vitamin it is deficient in the fat-soluble factor. They

found that in feeding experiments where all other requirements had been provided for, the diet of rats must contain 60 % of barley in order to get improved growth with a diet otherwise deficient in the fat-soluble factor.

Southgate [1924], who examined barley, kilned malt, unfined and fined beers for the A vitamin was unable to detect it in any of the materials examined.

From what is known of the solubility of the fat-soluble vitamins it is improbable that the process of macerating the malted grain with water would result in the extraction of any appreciable amount of them. After boiling the wort the almost clear liquid is drawn off and as large a surface as possible of the hot liquid exposed to the air so that efficient aeration may take place, the aeration being continued during the process of cooling. Great importance is attached by the brewer to the thorough aeration of the wort and there is no doubt that this process would be highly detrimental to any A vitamin present in the original liquid.

It seems more probable therefore that the A vitamin is synthesised directly by the yeast cell and that this synthesis is brought about in the absence of any direct solar radiation. This result, if definitely established, would be of interest since, in so many instances, solar energy has been shown to play an important part in the production of substances rich in the fat-soluble vitamin.

Ehrlich [1907] showed that the higher alcohols, which are derived from the amino acids of the nutrient protein, are only formed when carbohydrate fermentation is taking place; it is possible that the energy set free by the fermentation of the carbohydrate enables the yeast cell to carry out various metabolic processes, not the least important of which may be the synthesis of the fat-soluble vitamin.

We desire to express our indebtedness to the Food Investigation Board for a grant which has enabled this investigation to be carried out; to thank Miss D. G. Hoffert for assisting us in the work and to acknowledge the courtesy of Messrs Watney Coombe and Reid in supplying us with material.

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VIII. THE EFFECT OF IRRADIATION AND DIET ON CALCIUM AND PHOSPHORUS METABOLISM.

By JOHN McASKILL HENDERSON.

From the Rowett Institute, Aberdeen.

(*Received December 15th, 1924.*)

The metabolism of calcium and phosphorus is intimately involved in the action of ultra-violet irradiation. Hess and Lundagen [1922] have shown that in infants there is a seasonal tide of blood phosphate depending on the seasonal variation in sunlight. The increased deposition of calcium and phosphorus in bone as a result of irradiation, whether natural or artificial, has been amply proved by such work as that of McCollum and others [Powers, Park, Shipley, McCollum and Simmonds, 1921]; and numerous experiments have been carried out in investigating the effect of ultra-violet light on growth and the prevention of xerophthalmia.

In most of the above instances, however, the changes in calcium and phosphorus metabolism have been deduced from a final chemical analysis of the tissues, or from histological examination. In only very few cases has a complete calcium and phosphorus balance been followed out.

The present work was accordingly undertaken with the object of obtaining a quantitative estimate of the retention of these minerals under the influence of light over a fairly long period. Previous work, for the greater part, where it has not been on the human subject, has been carried out on small animals. For the present investigation the pig was selected for the following reasons:

(1) The growing pig of about three months old retains large amounts of calcium and phosphorus—roughly about 5 g. of CaO and of P_2O_5 per diem [Orr, 1923].

(2) A considerable amount of data on the retention of these minerals by the pig has already been accumulated at this Institute and serves as a guide.

(3) In the pig there is no thick coat to interfere with irradiation of the skin.

The importance of the proportions in which inorganic constituents are present in the diet has been shown by Elliot, Crichton and Orr in their work on rickets in pigs [1922]. In the case of the rat, Hess, Unger and Pappenheimer [1922] find that the rickets-producing effect of a diet adequate in calcium but low in phosphorus may be prevented by short exposures to direct sunlight. In the present investigation the calcium and phosphorus retention was accordingly determined in irradiated pigs which were on diets ill-balanced and

well-balanced respectively, particularly as regards their calcium, phosphorus and magnesium content. This aspect of the problem is of interest since the interrelation between ultra-violet light and vitamin A has already been carefully worked out by Hume [1922], Goldblatt and Soames [1923], Steenbock and Nelson [1923]. The problem, too, is not without its clinical significance in the treatment of rickets [Mellanby, 1924].

Two separate experiments were carried out. In the first, two pigs were used. One was irradiated and one kept in the dark. Both pigs were on a diet badly balanced as regards several of the mineral constituents. In the second experiment three pigs were used; one irradiated, one in darkness and one in diffuse light. All three were on a diet well-balanced as regards its mineral content.

Experimental.

Experiment 1. *Animals.* Two hog pigs of ten weeks old were selected from the same litter. They were closely comparable as regards condition, and at the beginning of the experiment each weighed 21·5 kg.

Housing. The pigs were kept in large metabolism cages from which urine and faeces could be collected separately. All sunlight entering the room traversed glass; further, the cages were rendered practically dark by means of green cloth canopies at a height of 9 inches above each cage and draped down the sides. There was no interference with ventilation. The animals were weighed every morning and were allowed to run about the animal room for half an hour for exercise.

Diet. The ration was as follows:

	g. per day	CaO %	P_2O_5 %	N %
Oatmeal	180	0·0819	1·048	2·101
Maize-meal	270	0·0188	0·526	1·511
Middlings	450	0·1245	1·528	2·261
$CaCO_3$	6	3·8898	—	—
Distilled water	*ad lib.*	—	—	—
Intake per 48 hours	—	8·2300 g.	20·366 g.	36·072 g.

A comparison of the mineral ratios of this diet and of sow's milk shows the diet to be ill-balanced with regard to several of its inorganic constituents. Thus:

	Sow's milk	Diet
$CaO : P_2O_5$	10 : 9	10 : 25
$CaO : MgO$	20 : 1	20 : 19

The content of the ration in fat-soluble vitamin is low.

Analysis. The metabolism cages were washed out with distilled water every morning. The excreta were collected over 48-hour periods and the analyses then carried out. Calcium was precipitated as oxalate and estimated volumetrically. A slightly modified Pemberton-Neumann method was adopted for phosphorus.

Irradiation. The source of light was a carbon arc lamp which took ·36 ampere at 45 volts. The pig was irradiated for 1 hour daily at a distance of 3 feet from the lamp.

The time during which the animals were in the cages may be divided into three periods. (1) A preliminary period of 10 days to accustom them to their environment and to determine their appetites. (2) A pre-period of 10 days during which analyses of the excreta were carried out. (3) An experimental period of 24 days during which the analyses were continued and the irradiation of the irradiated pig was in progress. The condition of the non-irradiated pig later in the experiment became so unsatisfactory that for the last few days an attempt was made to improve it by irradiation.

Forty-four days after the pigs had been put in the metabolism cages they were killed. Autopsies were done and also analyses of the blood and bones.

Table I. Exp. 1. *Average daily excretions and balances in grams.*

				Calcium (intake: 4·11 unless indicated thus *)				Phosphorus (intake: 10·18 unless indicated thus *)			
	Days	Wt. of faeces	Wt. of pig (kg.)	Urine	Faeces	Total	Balance	Urine	Faeces	Total	Balance
				Irradiated pig 1193.							
	1–10	522	21·9	0·09	3·56	3·65	0·46	1·51	6·79	8·30	1·88
Light	10–18	735	22·4	0·14	3·42	3·56	0·55	2·23	6·56	8·79	1·39
Light	18–24	513	21·7	0·23	1·73	1·96	1·78	2·48	4·68	7·16	2·54
Light	24–32	614	22·1	0·14	1·63	1·77	2·10*	2·76	4·10	6·86	2·65*
				Non-irradiated pig 1192.							
	1–10	488	22·0	0·08	4·02	4·10	0·01	1·31	7·08	8·39	1·79
	10–18	373	22·2	0·10	2·64	2·74	0·26*	1·86	4·98	6·84	0·58*
	18–24	290	22·5	0·10	2·53	2·63	0·35*	1·51	4·38	5·89	1·45*
Light	24–32	457	22·7	0·10	3·02	3·12	0·10*	2·34	5·49	7·83	0·15*

* Owing to pig 1192 latterly refusing to consume the whole ration the average daily intake for the last three periods was 3·00, 2·98 and 3·22 g. CaO; and 7·42, 7·34 and 7·98 g. P_2O_5.

Table II. Exp. 2. *Average daily excretion and balances in grams.*

				Calcium (intake: 11·94 CaO)				Phosphorus (intake: 12·77 P_2O_5)			
	Days	Wt. of faeces	Wt. of pig (kg.)	Urine	Faeces	Total	Balance	Urine	Faeces	Total	Balance
				Irradiated pig 1322.							
	1– 8	447	37·5	0·41	6·50	6·91	5·03	0·85	6·69	7·54	5·23
	8–16	510	40·2	0·46	7·06	7·52	4·42	0·87	7·12	7·99	4·78
Light	16–24	397	43·1	0·42	6·12	6·54	5·40	0·60	6·36	6·96	5·81
Light	24–32	463	45·2	0·31	6·81	7·12	4·82	0·69	7·14	7·83	4·94
Light C.L.O.	32–40	391	48·1	0·51	6·32	6·83	5·11	0·88	6·61	7·49	5·28
Light C.L.O.	40–50	398	51·6	0·56	6·52	7·08	4·86	0·87	6·73	7·60	5·17
				Non-irradiated pig—in darkness 1321.							
	1– 8	489	37·1	0·37	7·10	7·47	4·47	1·21	6·90	8·11	4·66
	8–16	459	38·4	0·30	7·77	8·07	3·87	1·40	7·84	9·24	3·53
	16–24	458	42·6	0·26	6·52	6·78	5·16	1·33	7·42	8·75	4·02
	24–32	378	45·1	0·27	6·24	6·51	5·43	1·04	6·53	7·57	5·20
	32–40	420	48·1	0·31	6·47	6·78	5·16	1·00	7·51	8·51	4·26
C.L.O. last 6 days	40–50	401	51·7	0·21	6·57	6·96	4·98	1·14	6·71	7·85	4·92
				Non-irradiated pig—in diffuse light 1323.							
	1– 8	445	33·8	0·51	7·75	8·26	3·68	0·58	7·82	8·40	4·37
	8–16	479	36·2	0·45	7·85	8·30	3·64	0·60	7·63	8·23	4·54
	16–24	423	38·9	0·28	7·10	7·38	4·56	0·78	7·11	7·89	4·88
	24–34	388	42·2	0·29	6·32	6·61	5·33	0·65	6·55	7·20	5·57

Results. Fig. 1 shows the calcium and phosphorus balances for each pig. The abscissae mark days, while the ordinates represent retention of CaO and P_2O_5 in g. per diem. Before irradiation was begun the CaO and P_2O_5 balances were fairly steady, the CaO having shown a slight decrease. Comparing the experimental with the pre-period in the irradiated animal the following points may be noted:

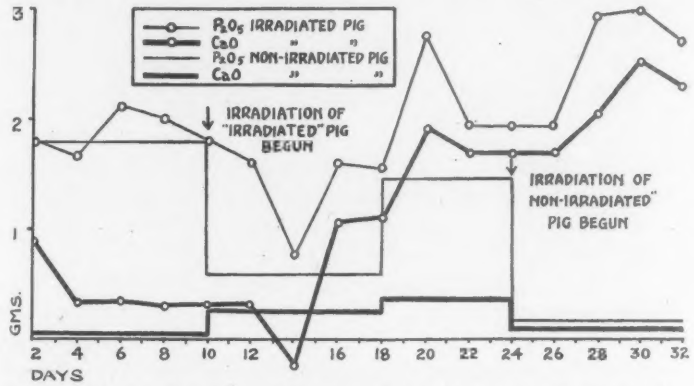

Fig. 1. Retention of calcium and phosphorus in irradiated and non-irradiated pigs.

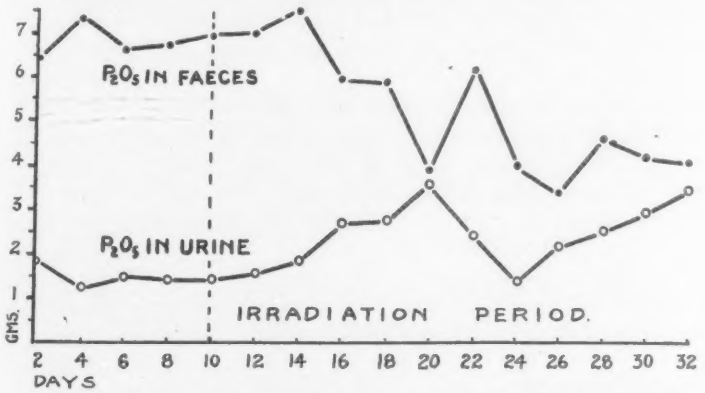

Fig. 2. Showing rise in urinary and fall in faecal excretion of P_2O_5 in irradiated pig.

(1) Both curves show a dip immediately after irradiation was begun, the pig actually reaching a negative calcium balance on the fourth day of irradiation. Thereafter there is a definite and steady rise. The rise is most marked in the case of the calcium retention. The average retention of CaO per diem during the pre-period was ·46 g.; during the irradiation period it rose to an average of 1·45 g. The corresponding figures for P_2O_5 retention were 1·88 g. and 2·07 g. respectively.

(2) The P_2O_5 and CaO balance curves approach each other in the experimental period and run a parallel course.

(3) The ratio of the urinary to the faecal excretion of calcium and phosphorus rises markedly during the experimental period. As regards the calcium, of the total CaO excreted in the pre-period 2·51 % was excreted in the urine; in the experimental period this figure rose to 6·94 %. An average of 18·21 % of the total P_2O_5 was excreted in the urine during the pre-period, and of 32·59 % during the experimental period. Fig. 2 shows the rise in urinary and fall in faecal excretion of P_2O_5 which occurred during the experimental period. It will be seen that, towards the end, the excretion in faeces and in urine was almost equal.

Non-irradiated Pig. After the tenth day of the experiment the non-irradiated pig suffered from gastro-intestinal disturbances, periods of diarrhoea and constipation alternating. As a result of these conditions balances became very irregular and it was impossible to graph 48-hour results. By averaging varying periods, however, it was obvious that the balances were gradually falling. This is not surprising in view of previous results [Husband, Godden and Richards, 1923] in which "pigs kept in metabolic cages and fed on a grain ration showed a progressively decreasing capability of absorbing and retaining CaO and P_2O_5." In the present case the average balances will be seen from Table I. Fig. 1 also shows the marked contrast between the rising retention of the irradiated pig and the falling retention of the non-irradiated pig. In the case of the latter, average periods are graphed: in the former, however, the balance from day to day is set out. The clinical picture corresponded with these findings, for the non-irradiated pig became less active than the irradiated, showed some loss of appetite and, on one occasion at least, vomited its food. In this condition irradiation, when applied for 10 days before the animal was killed, failed to have a beneficial effect; indeed, it possibly accelerated the downward trend. The balances at least became more irregular still. There was a rise in the ratio of urinary to faecal excretion with respect to both CaO and P_2O_5. Thus before irradiation 2·08 % of the CaO excreted appeared in the urine; after irradiation 3·36 % was thus excreted. As regards the P_2O_5, before irradiation 21·3 % was excreted in the urine; after irradiation this proportion rose to 29·8 %.

Table I shows the weight increments for the irradiated and for the non-irradiated pig. The weight gained over six weeks is remarkably small. When the pigs were put in the metabolism cages each weighed 21·0 kg. Forty days later, *i.e.* at the end of the experiment, the non-irradiated pig weighed 22·2 kg., and the irradiated pig 22·4. The latter, however, showed no signs of malnutrition.

Post-mortem Findings. The following are the results of the analyses of the blood and bones. In each case the right radius and ulna and the right tibia and fibula were removed and analysed separately.

	Irradiated Pig 1193		Non-irradiated Pig 1192		Pig 1168		Pig 1112	
	Tibia and fibula	Ulna and radius	Tibia and fibula	Ulna and radius	Tibia and fibula	Ulna and radius	Tibia and fibula	Ulna and radius
Wet weight of bones ...	140·99 g.		108·93 g.					
% ash in dry fat-extracted bone	45·86	43·39	39·77	41·23	43·00	43·92	39·48	39·77
% CaO in dry fat-extracted bone	24·25	23·35	20·84	22·17	21·28	22·86	20·73	21·02
% P_2O_5 in dry fat-extracted bone	20·32	18·39	18·12	17·39	18·71	19·51	15·36	16·92
Blood: CaO	12·9 mg. per 100 cc.		16·3 mg. per 100 cc.					
P_2O_5	138·4 „ 100 „		143·1 „ 100 „					

(Figures for two pigs, 1168 and 1112, from another experiment are placed here for comparison. These pigs showed definite clinical symptoms of rickets and on post-mortem examination there was "marked beading of the costo-chondral junctions" and "very marked beading" respectively, as also definite histological appearances of rickets.)[1]

In neither animal was any pathological condition found. Neither gross nor histological signs of rickets were observed in the bones. What appeared to be the only abnormality was an enlarged thyroid in the case of the non-irradiated pig.

Experiment 2. *Object.* Having obtained a definite effect of irradiation on the calcium and phosphorus balances when a badly balanced diet was being fed, it was determined to investigate the effects of irradiation, darkness and diffuse light when the animals were on a well-balanced ration.

Animals. Three comparable hog pigs, eleven weeks old were selected from the same litter.

Housing. The general conditions were identical with those in Experiment 1. As regards lighting, there were the following differences between the experimental conditions of the three animals.

(1) Pig 1321 was kept in a darkened metabolism cage and received no irradiation.

(2) Pig 1322 was kept under the same conditions, but had one hour's irradiation daily.

(3) Pig 1323 was kept in a metabolism cage without a canopy. That is, it was in the ordinary diffuse light of the animal room; it received no irradiation.

The pig in the dark and the irradiated pig occupied the cages for 66 days; the irradiation period of the latter was 42 days. The pig in diffuse light occupied the cage for 45 days.

Diet. The diet fed daily consisted of oatmeal, maize-meal, middlings—400 g. each. To this was added 50 cc. of a 10 % solution of NaCl; 20 g. $CaCO_3$, 40 g. blood-meal and 10 cc. olive oil. In the case of the irradiated pig and of the pig kept in the dark, 30 cc. of cod-liver oil was added to their daily ration from the 32nd and the 44th day respectively of the experiment.

[1] I am indebted to Dr J. P. McGowan for these specimens.

The intake per diem on the above ration was 11·94 g. CaO, 12·77 g. P_2O_5 and 28·83 g. N. The mineral ratios of this diet are roughly as follows:

	Diet	Sow's milk
$CaO : P_2O_5$	12 : 13	10 : 9
$CaO : MgO$	12 : 5	20 : 1

It is thus a better balanced diet than that employed in Experiment 1, particularly with respect to its calcium, phosphorus and magnesium content. NaCl and olive oil were added on account of their beneficial effect as indicated in some experiments at this Institute. The fat-soluble vitamin content of the ration is low. All experimental conditions were similar to those of Experiment 1.

In the case of the irradiated pig the analyses were carried out for 50 days. This was made up of an 8 days pre-period, and 42 days irradiation: during the last 18 days it was receiving in addition 30 cc. of cod-liver oil daily. Analyses of the excreta of the pig kept in darkness were conducted for 50 days, during the last six of which it received daily 30 cc. cod-liver oil. The analysis period of the pig in diffuse light lasted 34 days. Sixty-six days after being first put in the cages the irradiated pig and the pig in darkness were killed. Autopsies were done and analyses of the blood and bones.

Results. Table II (p. 54) shows the balance in the case of the irradiated pig. It will be seen that following irradiation there is a slight dip, but this is later compensated for. Despite oscillations, the balances during the irradiation period show no very definite tendency up or down. Since it was thought that the animal might be on a maximum retention of CaO and P_2O_5, 30 cc. of cod-liver oil was added to the daily diet on the 32nd day. There followed a depression and later an elevation of the balance: the average, however, remained practically the same as in the preceding period. In this connection it must be remembered that the animal had from the beginning of the experiment been receiving olive oil [Husband, Godden and Richards, 1923].

Table II gives also the average balances of the pig kept in darkness. On the whole they show no marked tendency to rise or fall. The cod-liver oil period towards the end is admittedly short, but sufficient to have shown a rise had such been striking. A characteristic feature of the table is the tendency of the P_2O_5 balance to fall below that of CaO. Further the P_2O_5 and CaO curves when graphed in detail show a decided departure from their general parallelism on the 20th and on the 42nd day. On these dates the CaO balance is raised and the P_2O_5 markedly depressed.

The parallelism of the calcium and phosphorus curves in this animal is not so striking as in the irradiated pigs of either Experiment 1 or 2, or as in the "diffuse light" pig of the latter experiment.

It will be noticed from Table II that the balances of the pig in diffuse light start at a slightly lower level than in the other two pigs. In this case, however, the tendency is undoubtedly upwards during the whole metabolic period with the P_2O_5 in general above the CaO.

The following table gives a rough comparative idea of the tendencies of

the balances in the three cases. It will be seen that while the intervals represent the pre-period, the period of irradiation alone, and the period of irradiation + cod-liver oil in the irradiated pig these merely correspond to arbitrary periods in the other animals. The table, however, serves the purpose of a broad summary.

Average retention of CaO *and* P_2O_5 *per day.*

	Irradiated pig		Pig in darkness		Pig in diffuse light		
Period	CaO	P_2O_5	CaO	P_2O_5	CaO	P_2O_5	Remarks
First 8 days	5·0	5·2	4·4	4·7	3·7	4·4	Pre-period for irradiated pig
Next 24 days	4·9	5·2	4·8	4·2	4·7	5·0	Light period for irradiated pig
Next 18 days	4·9	5·2	4·9	4·6	—	—	Irradiated pig, light: cod-liver oil. Darkness pig: 6 days cod-liver oil.

As regards the ratio of urinary to faecal excretion of CaO and P_2O_5 there is little to note. It varies within fairly narrow limits and shows no special tendencies.

The weight increments of all three pigs were comparable and show a steady rise (Table II). The irradiated pig put on, on the average, ·34 kg. per day, the pig in darkness ·35 kg. and that in diffuse light ·36 kg. The last, however, had been about 1·5 kg. lighter than the others at the beginning of the experiment.

Post-mortem Findings. The following are the results of the analysis of the blood and bones of the irradiated pig and that kept in the dark. In each case the left ulna and radius were used for analysis.

	Irradiated pig. Ulna and radius	Pig in darkness. Ulna and radius
Wet weight of bones	99·2 g.	101·9 g.
% ash in dry fat-extracted bone	55·65	52·48
% CaO in dry fat-extracted bone	29·57	28·06
% P_2O_5 in dry fat-extracted bone	21·44	19·90
Blood: CaO	9·43 mg. per 100 cc.	9·24 mg. per 100 cc.
P_2O_5	121·2 „ 100 „	98·5 „ 100 „

No abnormal condition was observed in either pig. Sections of the ribs showed normal calcification in each case.

Discussion of Results.

In considering the results of Experiments 1 and 2 the main difference is the marked contrast between the irradiated pig and its control in Experiment 1, and, on the other hand, the comparatively small differences in the second experiment between the irradiated pig, the pig in diffuse light and that kept in darkness. We are thrown back upon the conception that the balance of the diet is of fundamental importance [Orr, 1924]. When this is well adjusted the influence of light would seem to be at a minimum; for all three pigs in Experiment 2 grew normally, remained in good condition and, on the showing of their balance curves, retained adequate and fairly consistent amounts of calcium and phosphorus. On the other hand, in Experiment 1 neither pig grew, and the irradiated pig, which retained more calcium and phosphorus, alone remained in fair condition.

In the first experiment irradiation caused a marked rise in the retention of calcium and phosphorus but it is noteworthy that in each pig there was a slight dip in the balance curves following immediately upon irradiation; in the experimental pig a negative calcium balance was actually reached. Even in the irradiated pig of Experiment 2 there is a suggestion of a depression immediately after irradiation. This may be a specific effect of the light representing a negative phase or at least a period of re-adjustment. On the other hand the depressions of the curves in Experiment 1 were contributed to by an increased faecal excretion, for both pigs after irradiation suffered from diarrhoea for some days. Grant and Gates [1924] report cases of diarrhoea occurring among rabbits after irradiation.

Apart from stimulating the retention of calcium and phosphorus it appears that irradiation has a stabilising effect. Under its influence these minerals are probably retained in an optimum ratio. Both in Experiments 1 and 2 there is a well defined parallelism of the calcium and phosphorus retention curves during irradiation.

The relation of urinary to faecal excretion demands some notice. In the irradiated pig in Experiment 1 one of the most marked results of irradiation was the increase in urinary phosphorus and calcium. It will be seen from Fig. 2 that, while the total excretion was diminishing, the urinary excretion was steadily increasing. The same result, though in lesser degree, was obtained when in turn the non-irradiated pig was irradiated (Table II). There was, however, no such result following upon irradiation in Experiment 2. The increase in urinary excretion is a feature, then, of irradiation of the pig on a badly balanced diet; it is at least not marked when the diet is well balanced. In the treatment of rachitic children a similar result may be observed [Orr, *et al.*, 1923]. This increased excretion *via* the kidney would indicate that there has been on the part of the intestine a greater absorption, a diminished excretion, or both.

In view of recent work [Telfer, 1923] it is probable that the calcium and phosphorus of the faeces consist mainly of unabsorbed residues. If this be so, the effect of the irradiation is to cause an increased absorption by the bowel wall and thus present a greater amount of calcium and phosphorus to the organism. From this reasoning it is obvious that the increase in CaO and P_2O_5 absorbed by the bowel under the influence of light is even more than what is indicated by a graph of the total retention.

The increase in urinary excretion, particularly of phosphorus, also suggests that it is possible as the result of irradiation to induce absorption by the gut at a rate greater than the utilising mechanism can cope with. In the case of the non-irradiated pig in Experiment 1 there was, judging by the criterion of increased urinary excretion, increased absorption but little evidence of utilisation.

It might have been expected that even on a well-balanced diet and a maximum retention of calcium and phosphorus there would have been an

increase in the amount of these minerals in the urine following irradiation. Table II indicates that there was no increased absorption with rejection of the superfluous calcium and phosphorus. It may be that not only was the animal retaining a maximum but that in the circumstances a maximum was being absorbed by the wall of the gut. Thus neither irradiation nor cod-liver oil could cause any increase in absorption.

The failure of growth in the irradiated pig in Experiment 1 is striking. By many it is assumed that growth is essential to rickets [Parks, 1923] and it seems at first sight an attractive theory that irradiation may in part exercise its anti-rachitic effect through inhibiting undue general growth; that failure to grow is really a protective mechanism. There are, however, too many results showing growth under the stimulus of light to make this idea tenable. At the other extreme there are workers who, as a result of experiment, hold that the "growth factor" and the "anti-rachitic factor" are identical [Steenbock and Nelson, 1923]. In the present instance of the irradiated pig in Experiment 1 it is obvious that light may stimulate the retention of calcium and phosphorus without causing any concomitant increase in general body growth. Webster and Hill [1924] have recently pointed out an interesting example which, in their opinion, suggests that "the radiant energy necessary to promote growth appears to be other than that required to cure rickets." In the case of a rat which on irradiation showed no increase in growth over the controls, the P_2O_5 content of the carcass was nevertheless appreciably higher.

The analyses of the bones as detailed above reveals a difference in the direction one would have been led to expect. Thus, averaging the two sets of bones in Experiment 1, it is found that those of the irradiated pig contain 4·6 % more CaO and 3·2 % more P_2O_5 than those of the pig in darkness. As regards the corresponding pigs in Experiment 2 the figures are respectively 1·15 % and 1·14 %. That a terminal analysis of bones, however—unless it be the whole skeleton—gives only a rough indication of the amounts of CaO and P_2O_5 absorbed and deposited, is seen from the fact that had the ulna and radius alone been compared in corresponding pigs no marked difference would have been observable.

It will be noticed that in Experiment 2 the irradiated pig has a slightly higher blood calcium and a markedly higher blood phosphorus. The phosphorus retention in this pig had been greater than in the pig kept in darkness. But in Experiment 1 the control pig has a higher blood content of both minerals. It must be remembered, however, that this control had been irradiated for the previous 10 days and that while it was not retaining and depositing calcium and phosphorus it was absorbing considerable quantities as is shown by the urinary excretion of CaO and P_2O_5. It is not surprising that the blood calcium and phosphorus are high after irradiation, particularly in an animal which is not depositing these minerals. It is doubtful, however, if much can be deduced from the figures for the blood analysis. There are possibly in pigs marked individual differences in the CaO and P_2O_5 content

of the blood. Had it been possible the variation under light treatment would have been followed out in each individual pig but this was found incompatible with the success of the metabolic experiment since it was feared that the disturbance occasioned the pig by taking a blood sample might upset the balances.

Summary.

(1) In a pig on a diet badly balanced particularly with respect to phosphorus, calcium and magnesium (P_2O_5 : CaO : MgO—3 : 1 : 1) 1 hour's irradiation daily for 24 days with a carbon arc lamp definitely increased the calcium and phosphorus retention as compared with a control animal kept in the dark. The balance curves for these minerals tended to approach each other in the light period and run a parallel course.

(2) Following upon irradiation the calcium and phosphorus in the urine rose, both actually and relatively to their excretion in the faeces, suggesting an increased absorption from the gut or a decreased excretion.

(3) The above results took place in the irradiated pig without any concomitant increase in body weight. This would indicate that a stimulus to calcium and phosphorus retention does not necessarily involve a stimulus to growth in general.

(4) In pigs on a satisfactory diet, well balanced particularly with respect to calcium and phosphorus (P_2O_5 : CaO—1 : 1), darkness, diffuse light (through glass) and irradiation of 1 hour per day for 52 days caused little difference in the calcium and phosphorus retention, though such difference as there was, was in favour of the irradiated pig.

(5) In the irradiated pig on a well-balanced diet no tendency was observed for the urinary calcium and phosphorus to increase after irradiation.

(6) Analyses of the bones were in general agreement with the findings of the metabolic analyses.

I wish to express my indebtedness to Dr J. B. Orr, Director of the Rowett Institute, on whose suggestion the above work was undertaken; also to Dr H. E. Magee and Mr G. A. Reay for valuable assistance in the analytical work.

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IX. THE BACTERICIDAL ACTION OF THE BLOOD IN CERTAIN DIETARY DEFICIENCIES.

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In the present paper an attempt has been made to determine the effects of diets deficient in vitamins and in certain other essential factors on the bactericidal power of whole blood.

Zilva [1919] has shown that the power to form agglutinins, complement and immune body is not decreased in the rat as the result of diets lacking in vitamins, while Werkman [1923] has found that both in the rat and rabbit there is no inability to form agglutinins, precipitins, haemolysins and bacteriolysins on diets deficient in vitamins A and B. Findlay and Mackenzie [1922], confirmed by Werkman [1923], could find no defect in the opsonic power of the blood. Smith and Wason [1923] however stated that the bacteriotropic and bactericidal action of the serum on *B. typhosus* was reduced in rats fed on diets lacking vitamins A and B. In the case of guinea-pigs fed on a diet deficient in vitamin C, Findlay [1923] noted a diminished leucocytic response to bacterial infection, while in the case of pigeons lacking vitamin B the peritoneal exudate had less bactericidal power than that of control birds.

The present investigations have been carried out on the bactericidal power of the whole blood for *Staphylococcus aureus* in normal rats and in rats subjected to the following procedures:

A. Fed on a diet lacking vitamin A and the antirachitic factor.

B. Fed on a diet lacking vitamin A and the antirachitic factor and exposed to ultra-violet light.

C. Fed on a diet lacking the antirachitic factor and phosphorus.

D. Fed on a diet lacking vitamin B.

The rats used in these experiments were all bred from laboratory stock. As preliminary experiments had shown that the employment of anaesthetics in killing the animals caused variations in the bactericidal power of the blood it was decided to kill the rats by a blow on the back of the head; the thorax was then opened and the blood was removed from the heart with aseptic precautions. The rats used as controls were fed on laboratory diet with the addition of cod-liver oil and marmite. All the animal experiments were carried out by one of us (G. M. F.). The observations on the bactericidal power of the blood were made by the slide cell method of Wright, Colebrook and Storer [1923].

A. Rats fed on a diet deficient in vitamin A and the antirachitic factor.

The rats used in this experiment were placed on the following diet:

Caseinogen	14 g.		
Starch	77		
Marmite	5		
Inorganic salt mixture:			
NaCl	0·32 g.	$CaH_4(PO_4)_2.2H_2O$	1·02 g.
$MgSO_4$	0·56	Ca lactate ...	2·46
$NaH_2PO_4.2H_2O$	0·66	Fe citrate ...	0·22
K_2HPO_4 ...	1·76		

Though lacking both vitamin A and the antirachitic factor, animals placed on this diet do not develop rickets since the proportions of calcium and phosphorus are correct.

In the first experiment the rats were allowed to continue on the diet until they began to show symptoms due to a deficiency of vitamin A. The body temperature at the time of death and the bactericidal power of the blood are shown in Table I.

It will be seen that the body temperature of the normal rat varies from about 37·0° to 38·0°, while the percentage of staphylococci killed by the blood is as a rule about 70. There is thus a definite fall in the bactericidal power of the blood in association with a deficiency of vitamin A and the antirachitic factor, but only after the onset of keratomalacia or broncho-pneumonia.

In the second experiment young rats were placed on the diet deficient in vitamin A and the antirachitic factor; they were then killed after varying periods on the diet, a random selection being made. Here again, as shown in Table II, there is no fall of bactericidal power until after the onset of keratomalacia.

B. Rats fed on a diet deficient in vitamin A and the antirachitic factor, but exposed to ultra-violet light.

The rats were placed on the same diet as in the previous experiments but were irradiated for 20 minutes each day with a mercury vapour lamp at a distance of 8 inches.

The experiments of Colebrook, Eidenow and Hill [1924] have shown that when a healthy animal is exposed to ultra-violet light there results an increase in the bactericidal power of the blood: this increase lasts for at least 2 hours after the exposure but tends to disappear 4 hours after exposure. The rats were killed after varying periods on the diet and, except in two cases, from 6 to 24 hours after exposure to the ultra-violet light. It will be seen from Table II that the bactericidal power of the blood of the animals exposed to

light did not fall in the same way as in those kept in diffused daylight; this must be correlated with the fact that the animals exposed to ultra-violet light did not develop keratomalacia or other intercurrent infections so rapidly as the non-exposed rats. The growth curves also of the irradiated rats were better than those of the non-exposed rats, a confirmation of the findings of Hume [1922] and Goldblatt and Soames [1922].

Table I. *The Bactericidal Power of the Blood in Control Rats and in Rats on a Diet deficient both in Vitamin* A *and the Antirachitic Factor.*

No. of rat	Body temperature at death	Percentage of staphylococci killed	Remarks
		Vitamin-rich diet.	
1	37·7	72	—
2	37·1	65	—
3	37·4	67	—
4	37·3	74	—
5	37·2	73	—
6	37·6	76	—
7	37·5	68	—
Average	37·4	71	—
		— Vitamin A *and — Antirachitic Factor.*	
8	37·2	67	− Keratomalacia
9	37·2	46	+ ,,
10	37·7	60	− ,,
11	37·2	71	− ,,
12	36·9	50	− ,, / + Broncho-pneumonia
13	37·6	65	+ Keratomalacia
14	36·5	43	+ ,,
15	37·1	38	+ ,,
16	37·2	53	+ ,,
17	37·1	45	+ ,,
18	37·2	55	+ ,,
19	37·5	57	− ,, / + Broncho-pneumonia
Average	37·2	54	—
		— Vitamin A *and — Antirachitic factor. Single exposure to Ultra-violet Light* 1 *hour before death.*	
20	37·2	48	+ Keratomalacia
21	35·0	47	+ ,,
22	37·1	45	+ ,,
23	37·2	54	+ ,,
Average	36·6	48·5	—

Four rats suffering from keratomalacia were exposed to ultra-violet light for 30 minutes and the bactericidal action of the blood tested 1 hour later. As shown in Table I the bactericidal action of the blood in these animals was still low after irradiation. This is in agreement with the observations of Colebrook [1924], who found that patients with a low bactericidal power of the blood do not show any increased bactericidal action of the blood after exposure to ultra-violet light.

Table II. *The Bactericidal Power of the Blood of Rats on a Diet deficient both in Vitamin* A *and the Antirachitic Factor and killed after varying periods on the Diet.*

No. of rat	No. of days on diet	Body temperature at death	Percentage of staphylococci killed		Remarks
		Vitamin-rich diet.			
24	—	37·4	78		—
25	—	37·6	79		—
26	—	37·8	76		—
27	—	37·2	76		—
28	—	37·1	67		—
29	—	38·0	70		—
30	—	37·4	75		—
Average	—	37·5	74		—
		— Vitamin A *and — Antirachitic factor.*			
31	7	37·6	75	–	Keratomalacia
32	7	37·9	78	–	,,
33	7	37·7	77	–	,,
34	33	36·7	43	+	,,
35	33	35·4	38	+	,,
36	54	36·7	47	+	,,
37	54	36·8	45	+	,,
Average		37·0	58		—
		— Vitamin A *and — Antirachitic factor. Daily exposure to Ultra-violet Light.*			
38	7	37·2	78	–	Keratomalacia
39	7	37·4	77	–	,,
40	7	37·6	76	–	,,
41	26	37·3	90	–	,, killed 1 hour after exposure to ultra-violet light
42	26	37·0	92	–	,, killed 1 hour after exposure to ultra-violet light
43	33	37·8	75	–	,,
44	33	38·0	71	–	,,
45	54	37·7	69	–	,,
46	54	38·4	72	–	,,
47	78	38·0	72	–	,,
48	78	38·0	69	–	,,
49	92	36·9	54	+	,,
Average	—	37·7	74		—

C. Rats fed on a diet lacking the antirachitic factor and phosphorus.

When young rats are placed on the following diet [Pappenheimer, McCann and Zucker 1922]:

White flour	...	...	80·9 g.		
Egg albumin	...	...	10·0		
Butter fat	...	...	5·0		
Salt mixture:					
KCl	...	0·850 g.	Ca lactate	...	2·000 g.
Na_2CO_3	...	0·850	Fe citrate	...	0·100
$MgCO_3$	...	0·286	KI	...	0·0002

they invariably develop rickets. The diet is adequate for full growth since it contains both vitamins A and B; it can be rendered antirachitic by the

addition of cod-liver oil or phosphates or by exposing the animals to ultra-violet light. The rats in this experiment were allowed to remain on the diet for 3 weeks before testing the bactericidal power of the blood. All the rats had well-developed rickets. Reference to Table III shows that in two cases the bactericidal power of the blood was normal while in four cases it was greatly reduced.

Table III. *The Bactericidal Power of the Blood of Rats on a Diet deficient in the Antirachitic Factor and Phosphorus.*

No. of rat	No. of days on diet	Body temperature at death	Percentage of staphylococci killed
	Vitamin-rich diet.		
50	—	37·4	68
51	—	37·2	74
52	—	37·6	76
53	—	37·8	67
Average	—	37·5	71
	Rickets Rats.		
54	21	36·9	30
55	21	37·2	20
56	21	37·2	48
57	21	37·5	74
58	21	36·8	70
59	21	36·8	30
Average	—	37·1	45

Table IV. *The Bactericidal Power of the Blood of Rats on a Diet deficient in Vitamin* B.

No. of rat	No. of days on diet	Body temperature at death	Percentage of staphylococci killed
	Vitamin-rich diet.		
60	—	37·7	75
61	—	37·1	65
62	—	37·1	67
63	—	37·3	72
64	—	37·2	73
Average	—	37·3	70
	– Vitamin B.		
65	25	35·6	42
66	25	35·4	50
67	32	35·1	38
68	32	35·1	33
69	35	<35·0	38
70	35	<35·0	39
71	39	35·2	50
72	39	<35·0	50
73	41	36·2	51
Average	34	35·3	43

D. Rats fed on a diet lacking vitamin B.

In this experiment the rats were fed on the following diet:

Caseinogen	14 g.		
Starch	77		
Cod-liver oil	5		
Inorganic salt mixture:			
$NaCl$	0·32 g.	$CaH_4(PO_4)_2.2H_2O$	1·02 g.
$MgSO_4$	0·56	Ca lactate ...	2·46
$NaH_2PO_4.2H_2O$	0·66	Fe citrate ...	0·22
K_2HPO_4 ...	1·76		

The length of time on the diet, the body temperature and the bactericidal action of the blood are shown in Table IV. A fall in the bactericidal action of the blood occurs as the result of a diet deficient in vitamin B, apart from the occurrence of any bacterial infection, though the lowering of the bactericidal power in this type of deficiency is always associated with a fall in body temperature. In the case of animals fed on diets deficient in vitamin B, this decline in body temperature takes place some days before death.

Discussion.

The question naturally arises as to whether the bactericidal power of whole blood is due to the action of the leucocytes or the serum. Smith and Wason [1923], as previously indicated, found that the bactericidal power of the serum against *B. typhosus* was decreased as the result of diets deficient in vitamins A and B. It is doubtful whether any great significance can be attached to their results, as the bactericidal action on *B. typhosus* of the serum of normal rats is known to vary very greatly. The blood of wild and black rats as a rule has no bactericidal action on *B. typhosus*, while that of white rats has a slight bactericidal action.

The serum of the rat has little or no bactericidal action on *Staphylococcus aureus*.

The work of Wright, Colebrook and Storer [1923], on the other hand, has proved that the bactericidal power of whole blood against *Staphylococcus* is largely due to the polymorphonuclear leucocytes.

Any factor which tends either to reduce the numbers of the polymorphonuclear leucocytes or to impair their functional activity might therefore decrease the bactericidal power of the blood. It is thus of interest to determine whether there is any reduction in the number of the polymorphonuclear leucocytes in the blood in association with vitamin deficiencies.

Total and differential white cell counts were made in 10 normal rats and in 10 rats with keratomalacia as the result of a diet lacking vitamin A. The following figures were obtained:

Control rats:	9450 leucocytes per mm.^{3}	Polymorphs	35 %
– Vitamin A rats:	8170 „ „ „	„	48 „

There is thus little difference in the total leucocyte counts, while in the case of the vitamin A deficient rats there is a polymorphonuclear leucocytosis, probably as a result of the bacterial infection. Cramer, Mottram and Drew [1921] have obtained similar figures. When rats are fed on diets deficient in vitamin B, there is a fall in the total leucocyte count, but this fall only affects the lymphocytes, the polymorphs, as shown by Cramer, Mottram and Drew [1921] exhibiting little or no decrease in number. It is obvious therefore that the fall in bactericidal power of the blood in vitamin A and B deficiencies cannot be due to a decrease in the number of polymorphonuclear leucocytes.

There is, however, a possibility that the killing power of all the polymorphonuclear leucocytes in the blood is not equal. If young and aged polymorphonuclear leucocytes are of less vigour than adult cells, then the bactericidal power of the blood should tend to decrease whenever there is an excessive proportion of very young or very old polymorphs in the blood stream, unaccompanied by a definite leucocytosis. An excessive number of aged polymorphonuclear leucocytes for instance would survive in the blood stream in a condition where there is diminished leucoblastic activity of the bone marrow unassociated with bacterial infection. Such a condition is found in animals fed on diets deficient in vitamin B, where the bone marrow, as shown by Shipley, McCollum and Simmonds [1921], exhibits numerous small haemorrhages. In addition Happ [1922] has found that in rats suffering from a deficiency of vitamin B there is a very high proportion of aged or moribund polymorphs in the blood stream.

Conclusions.

1. The blood of normal rats, tested against *Staphylococcus aureus*, shows a killing power of about 70 %.

2. Rats fed on a diet deficient in vitamin A and the antirachitic factor show a reduction in the bactericidal power of the blood only after the onset of keratomalacia or some other infection.

3. In rats fed on a diet deficient in vitamin A and the antirachitic factor, but exposed to ultra-violet light, the onset of bacterial infection and the reduction in the bactericidal power of the blood are delayed.

4. Rats suffering from keratomalacia as the result of a diet deficient in vitamin A continue to show a low bactericidal power of the blood after exposure to ultra-violet light.

5. Rats fed on a diet lacking phosphorus and the antirachitic factor show, in most cases, a reduction of the bactericidal power of the blood.

6. Rats fed on a diet lacking vitamin B show a reduction in the bactericidal power of the blood unassociated with bacterial infection but possibly correlated with the fall in body temperature.

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X. THE DETERMINATION OF CYANATES IN BLOOD.

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The occurrence of cyanic acid during the oxidative deamination of glycine, alanine and sarcosine has been quoted recently in support of the hypothesis that this acid is an important intermediate product in the formation of urea in the animal body [Fearon and Montgomery, 1924]. Additional support was claimed from the work of Nicloux and Welter [1922], as reviewed by Riggs in *Chemical Abstracts* [1923].

At the time the original paper was not available, but a subsequent study of the work revealed the unexpected fact that Nicloux and Welter do not claim to have obtained any positive evidence for the occurrence of cyanic acid in the blood of the dog. Their data, however, are also capable of the interpretation given by Riggs. "Ten parts of plasma of dog blood mixed with 1 part of satd. NH_4Cl soln. and incubated 36 hrs at 35° showed an increase of about 5 % in the urea content over that of the initial plasma. By adding 0·1 mg. KCNO to 1 cc. of plasma-NH_4Cl mixture, the increase of urea in 36 hrs was 21 %. Cyanates therefore occur in blood" [Riggs, 1923].

With the object of removing this ambiguity, the blood of the rabbit and the cat was examined for cyanate by a method similar to that of Nicloux and Welter with slight modifications.

In several animals a positive result was obtained. For the rabbit the cyanate value was found to be 0·7 mg. per 100 cc. plasma; and for the cat a value of 1·0 mg. per 100 cc. plasma was obtained, expressed as HOCN.

The Method of Determining Cyanate in Blood.

This can most readily be accomplished by converting the cyanate into urea by incubation with a suitable ammonium salt solution in accordance with the equation:

$$NaOCN + NH_4Cl = CON_2H_4 + NaCl.$$

This change is not quantitative as some of the cyanate undergoes hydrolysis to ammonia and carbon dioxide, depending on the time and temperature of the incubation, and the concentration of the added ammonium salt. Nicloux and Welter found that a 0·1 % solution of KCNO, incubated at 35° with a saturated solution of NH_4Cl, gave 36 % of the possible urea in 12 hours, and 77 % in 48 hours.

Recovery of cyanate as urea. 20 cc. of a fresh KOCN solution (0·101 %) were incubated with 0·2 g. $(NH_4)_2SO_4$ at 40° for 24 hours. The urea recovered represented 77·1 % of the total cyanate added.

For the determination of the urea the xanthydrol method was applied as in the previous paper [Fearon and Montgomery, 1924]. The more delicate urease methods of estimating urea are not so suitable, since Fearon [1923] and Mack and Villars [1923] have shown the formation of cyanic acid during the decomposition of urea by urease; consequently confusion might arise between any cyanic acid present in the plasma and cyanic acid formed by the influence of the enzyme.

Details of method. 30 to 40 cc. of plasma were obtained by centrifugalisation of the fresh blood and divided into two equal samples, A and B. A, the control, was treated with toluene and incubated in a sterile tube for 24 hours at 43°. 0·2 g. of ammonium sulphate was added to B, which was incubated for the same time under the same conditions. An equal volume of concentrated Tanret's reagent[1] was then added to each tube, and the contents filtered. The filtrates were treated with equal volumes of glacial acetic acid, and 1 cc. of a 10 % solution of xanthydrol in methyl alcohol.

At the end of 12 hours the precipitates of dixanthylurea were filtered off, washed with 3 cc. absolute alcohol saturated with dixanthylurea, dried and weighed, and the weight of the urea present determined by dividing the weight of the dixanthylurea by 7.

The solubility of the dixanthylurea in the acetic acid mixture under the conditions of the experiment was determined and found to be 1·6 mg. per 100 cc.

Table I. *Recovery of urea added to 5 cc. of blood plasma.*

Exp. 1.	Initial urea content of plasma	2·23 mg. per 5 cc.
	Final urea content of plasma	3·74 „ „ 5 „
	Estimated amount of urea added ...	1·51 „ „ 5 „
	Actual amount of urea added	1·56 „ „ 5 „
Exp. 2.	Initial urea content of plasma	1·53 mg. per 5 cc.
	Final urea content of plasma	3·68 „ „ 5 „
	Estimated amount of urea added ...	2·15 „ „ 5 „
	Actual amount of urea added	2·20 „ „ 5 „

Table II. *Cyanic acid concentration of rabbit plasma.*

Rabbit. Three hours after meal of 130 cc. of milk. Urea determined in 10 cc. of plasma.

Urea content of plasma A	55·3 mg. per 100 cc.
„ „ „ „ B	56·4 „ „ 100 „

Increase represents 0·78 mg. cyanic acid per 100 cc.

Table III. *Cyanic acid content of cat plasma.*

Urea determinations in 12·5 cc. of plasma before and after incubation with ammonium sulphate.

Cat I.	Urea content of plasma A	63·3 mg. in 100 cc.
	„ „ „ „ B	64·7 „ „ 100 „
	Urea increase represents 1·04 mg. cyanic acid in 100 cc.	
Cat II.	Urea content of plasma A	61·3 mg. in 100 cc.
	„ „ „ „ B	63·17 „ „ 100 „
	Urea increase represents 1·05 mg. cyanic acid in 100 cc.	

[1] Tanret's reagent: mercuric chloride (2·71 g.), potassium iodide (7·2 g.), in 66·6 cc. of glacial acetic acid, made up to 100 cc. with distilled water.

By means of control experiments the limits of error of the method were found to lie within ± 0·2 mg. per 100 cc.

Confirmatory test for cyanate in blood plasma. To determine if the increase in urea due to incubation with the ammonium salt arose from pre-existing cyanate or from some other source, such as the hydrolysis of some unidentified ureide, a determination was carried out on rabbit blood plasma after acidification by the addition of 3 cc. $N/10$ H_2SO_4 to 15 cc. plasma. This would destroy any cyanate present by converting it into free cyanic acid which is rapidly hydrolysed in acid solution. The urea content of this plasma is shown in the following table:

Table IV. *Urea content of acidified plasma after incubation for* 24 *hours.*

Urea content of plasma	A	...	...	56·6 mg. per 100 cc.
„ „ „ „	B	...	...	56·7 „ „ 100 „

This confirms the belief that the increase in plasma urea after incubation with ammonium salts is due to the presence of cyanate, since previous acidification of the plasma checks the formation of urea.

Significance of Cyanic Acid in the Blood.

Since it is so closely related to urea, the occurrence of cyanic acid in the plasma suggests that it is either a precursor or a product of the urea formed in the organism. It may represent amino-nitrogen on its way to be converted into urea, or it may result from the enzyme dissociation of urea. The evidence available is strongly in support of the former view. There is at present no ground for the belief that the enzyme urease is present in the tissues of the human body, and considerable indirect evidence that it does not occur. Perfusion of surviving dog's liver with dog's blood, saline, and urea (up to 4 g. per litre) showed no increase in ammonia; consequently, Wakeman and Dakin [1911] concluded that the formation of urea appeared to be an irreversible reaction. Luck [1924] has recently demonstrated the presence of urease in the gastric mucosa of the Carnivora and Ruminantia, but this may be due to ferments taken in with the food or arising from micro-organisms in the food. The possibility of extraneous infection must always be borne in mind in working with urea as Werner [1918] has shown; the dissociation of urea in aqueous solution at low temperature is due, probably entirely, to infection. The number of micro-organisms capable of decomposing urea is very large.

The occurrence of a variable amount of cyanate in plasma will also explain the varying values for blood ammonia obtained by different observers. Solutions of cyanate in low concentration may be completely hydrolysed to ammonia and carbon dioxide by prolonged aspiration at room temperature; and the earlier and more drastic methods for estimating blood ammonia no doubt owe their high values to the unrecognised presence of cyanate.

Conclusions.

1. Cyanic acid is present in the blood plasma of the cat to the extent of 1·04 mg. %, and in the blood plasma of the rabbit to the extent of 0·78 mg. %, some hours after a protein meal.

2. Its presence is considered to be of significance in the formation of urea in the animal body.

3. The varying results found in the determination of the blood ammonia may be due to the presence of cyanic acid.

I wish to acknowledge my indebtedness to Prof. H. Pringle of this department for facilities afforded in this research.

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XI. A POSSIBLE CORRELATION BETWEEN DIETARY PROTEIN AND LOSS OF FUR IN YOUNG GROWING RATS.

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The importance of protein in the diet has been emphasised by numerous workers. It is impossible here to give even a brief survey of the literature on this subject, but reference might well be made to the interesting and critical paper of Mitchell [1924], who takes a broad view of protein metabolism and gives a considerable number of references.

Loss of hair as a result of a special diet has been noted by several observers. McCollum, Simmonds and Parsons [1918] comment on this phenomenon in rats when the animals were fed on dried potato and butter fat, supplemented by the inorganic elements sodium, chlorine and calcium, in which these observers state that the potato is deficient.

Boas [1924, 1] also reports a baldness in rats fed on a commercial preparation of egg-white as the sole source of protein. Her animals, at the same time, ceased to gain weight; they remained stationary for a week or so and then lost weight. With alterations in the diet, the animals recovered, gained weight again, and new hair appeared in less than a week. Thus the loss of fur was obviously dietetic in origin. In a more recent paper Boas[1] states that no cessation of growth or loss of fur occurs when the rats received the white from fresh English eggs as the sole source of protein [1924, 2].

A loss of hair in rats was commented on by the author in a previous paper [1924]. The animals were fed on a diet of white bread, butter and salt mixture. A loss of hair was noticed when the young rats were about 6 weeks old, *i.e.* after about 3 weeks on the special diet. Eventually the rats' hair grew again *on the same diet*, although not so rapidly as when the diet was altered, *e.g.* when caseinogen was added. This rapid improvement on the addition of caseinogen is quite in agreement with the findings of Boas [1924, 1].

A diet of bread, butter and salt mixture might be regarded as poor from the point of view of protein, and although the rats showed no signs of lack of vitamin B during the $7\frac{1}{2}$ months of the experiment, it might be suggested that the vitamin B content was at a minimum. Thus two possible dietetic factors

[1] I am indebted to Boas for allowing me to read this paper in proof form.

might be responsible for the loss of fur in the young rats. In the same communication [1924] it was shown that rats fed on a similar diet, with the addition of wheat germ extract, lost their fur in the same manner, so that the addition of more vitamin B to the diet was ineffective. This has been confirmed again by using a 3 % extract of vitamin B made from marmite, instead of wheat germ extract, when the loss of fur occurred just as before. This is also proved by the experiments of Boas, for her rats which showed loss of fur were receiving appreciable quantities of marmite in their diet. It seemed, therefore, highly probable that the deficiency must be looked for in either the quality or quantity (or both) of the protein. The experiments described in this paper were started in the hope of throwing some light on this problem.

Experimental.

1. *Animals.* The rats used in this research were piebald animals bred in the laboratory [Hartwell, Mottram and Mottram, 1923]. Twelve rats were used for each experiment, 6 ♀ and 6 ♂, and were kept in a large cage together. They were weighed once a day for the first few weeks and afterwards once a week.

2. *Foods.* The animals were given food *ad lib.*; all constituents were well mixed so that the rats could not pick out any one substance. The food was given as a moist paste. In hot weather the rats needed extra water to drink, but in cold weather they drank none.

The diets given were:

1. 30 g. white bread, 3 g. butter, 0·7 g. salt mixture, 60 cc. 3 % extract containing vitamin B [Hartwell, 1922, 2].

2. 30 g. brown bread, 3 g. butter, 0·7 g. salt mixture, 60 cc. water.

3. 30 g. white bread, 3 g. butter, 0·7 g. salt mixture, 60 cc. water, 1·5 g. food casein [Hartwell, 1922, 1].

4. 30 g. white bread, 3 g. butter, 0·7 g. salt mixture, 60 cc. water, 1·5 g. gluten[1] (80·62 % protein).

5. 30 g. white bread, 3 g. butter, 0·7 g. salt mixture, 84 cc. water, 1·5 g. gelatin (the gelatin was soaked overnight in 18 cc. of cold water).

6. 100 g. oatmeal (medium), 500 cc. water, 2·88 g. salt mixture, 14 g. butter. The oatmeal was cooked for 1½ hours in 400 cc. water, then 100 cc. more water were added together with the butter and salt mixture, and the whole well mixed.

7. 100 g. boiled potato, 25 cc. water, 1 g. salt mixture, 5 g. butter.

Results.

The composite growth curves of the various animals are shown in the figure. The curves of the male animals only are given, the reason being that the does became pregnant at different dates, so that their curves are not strictly comparable one with the other. Growth is only given for 14 weeks,

[1] I am indebted to Callard & Co. for supplying me with an analysis of the gluten used in this work.

although some of the experiments lasted for a year, and some are still in progress. That there is no deficiency in vitamin B in any of these diets is proved by the continuous, though in some experiments slow, growth for this period. The work is being continued with the view to ascertaining the effect of these various diets on gestation, and results of this will be given in a later paper.

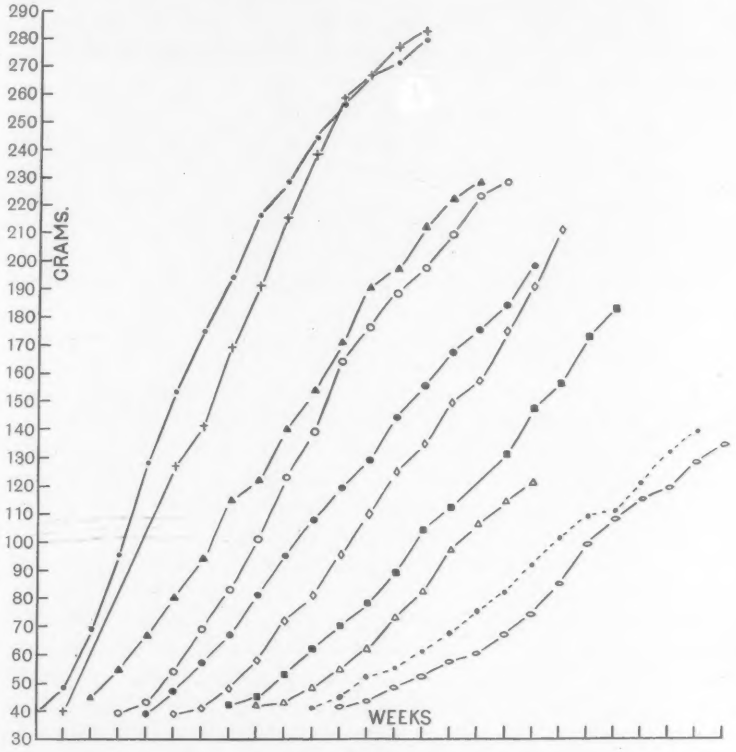

Fig. 1.

• ——— • Control animals. Good mixed diet (kitchen scraps, together with bread and milk).
\+ ——— + Diet of white bread, butter, salt mixture and food casein.
▲ ——— ▲ Diet of oatmeal (medium), butter and salt mixture.
○ ——— ○ Diet of white bread, butter, salt mixture and gelatin.
● ——— ● Diet of white bread, butter, salt mixture and gluten.
◇ ——— ◇ Diet of brown bread, butter and salt mixture.
■ ——— ■ Diet of white bread, butter, salt mixture and extract containing vitamin B made from marmite.
△ — — △ Diet of white bread, butter, salt mixture and wheat germ extract. (Taken from Hartwell [1924].)
• - - - - • Diet of white bread, butter and salt mixture. (Taken from Hartwell [1924].)
⊂ ——— ⊂ Diet of boiled potato, butter, and salt mixture.

From examination of the curves, it is obvious that all the animals grew, but at very different rates. The fur came off when the animals were fed on the potato and white bread diets, even when wheat germ extract or extract made from marmite containing vitamin B was added to the bread. With the potato diet, the fur grew again, but *very* slowly, and the new coat was poor. Old potatoes were used in this experiment but it is being repeated with new ones to see if there is any difference. With the bread diets, the fur also grew again, and when grown was in good condition. The growth of fur was more rapid than with the potato-fed rats, but not nearly so quick as when caseinogen was added to the bread diet.

Comments.

From these experiments it appears that the slow rate of growth is correlated with the falling of the animals' hair, but this may be mere coincidence. If the loss of fur in Boas' [1924, 1] experiments is due to the same dietetic error the slow rate of growth does not necessarily coincide with the falling hair, because her animals were growing more rapidly than mine.

It is interesting that the falling of the hair could be entirely prevented by adding more gluten to the bread diet, hence gluten in itself cannot be such a poor protein as is frequently claimed. Also the addition of extra gluten causes a marked improvement in the rate of growth. Gelatin and caseinogen also prevented the fur from coming out, the substitution of brown instead of white bread was similarly effective. From this latter fact alone, the casual observer would remark that the improvement must be due to the greater vitamin B content of brown bread, but that this is an incorrect assumption has been proved by adding wheat germ extract or extract containing vitamin B made from marmite to the white bread diet, when no improvement was obtained. Brown bread actually contains less protein than white bread [Plimmer, 1921], when calculated as dry constituents, but the difference is so slight as to be practically negligible. It is probable that the germ protein is different in composition from the endosperm protein, and although the germ is only a small part of the whole grain, its protein percentage is high [Osborne and Mendel, 1919; "crude protein" 40·25 %]. The author has not been able to find any analyses of the germ protein and therefore this explanation is put forward somewhat tentatively. It is, however, conceivable that some amino acid is absent from the endosperm protein, though present in the germ protein, so that the latter is highly effective in supplementing the former.

Boas [1924, 1] found that 5 g. of spinach per day per rat effected a cure, and although this may appear to be a small amount considering the percentage of water in green vegetables, yet the biological value of spinach protein is high [Lusk, 1917]. It is interesting to note that oatmeal protein is effective in promoting quite good growth, though not up to the control rate.

From the experiments described in this paper, it is justifiable to conclude that the quality or quantity (or both) of the dietary protein is a factor influencing the loss of hair in young growing rats. It is quite possible that a similar condition might be brought about by other dietary deficiencies.

Conclusions.

1. The loss of fur in a growing rat is closely correlated with the quality or quantity (or both) of the dietary protein.

2. This loss of fur can be remedied by adding more or better protein to the animals' diet.

3. A loss of fur was obtained when potato or white bread was used as sole source of protein. The addition of gluten, gelatin, or caseinogen, to the bread diet completely prevented the loss of fur. Brown bread or oatmeal as sole source of protein resulted in no loss of hair.

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XII. THE OSMOTIC PRESSURE OF HAEMOGLOBIN.

AN EXPLANATION OF THE EFFECT OF ACETIC ACID, BASED ON DONNAN'S THEORY OF MEMBRANE EQUILIBRIUM.

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In a recent publication [Wilson, 1923] on "The Effect of Carbon Dioxide and Acetic Acid on the Osmotic Pressure of Haemoglobin," it was shown that "the osmotic pressure of solutions of purified haemoglobin is three or four times as great when dialysed against acetic acid or water saturated with CO_2 (both about p_H 4) as against water alone." Reasons were given for believing that "this is due to the formation of some sort of ionising salt of haemoglobin, either one which ionises into several protein ions, or into protein and acetate or protein and bicarbonate ions" (p. 70). It was pointed out that a study of the hydrogen-ion concentration of the haemoglobin solution inside the osmometer and the fluid outside the osmometer should determine which kind of dissociation had taken place. If the ionisation were into protein ions only, the Donnan equilibrium [1911] should not come into play and the hydrogen-ion concentration of the inner and outer solutions in the osmometer should be the same when equilibrium was reached. If a haemoglobin acetate were formed which dissociated into haemoglobin and acetate ions, then the Donnan equilibrium would, presumably, occur and the inner solution would be less acid than the outer. The latter hypothesis is substantiated by the experiments to be reported in this paper. The conclusion is, therefore, drawn that the increased osmotic pressure observed is due to the formation of haemoglobin acetate. The resulting equilibrium relations have been quantitatively considered.

Experimental.

The haemoglobin[1] was several times recrystallised according to the method of Dudley and Evans [1921], which depends on the fact that reduced haemo-

[1] The haemoglobin used in these experiments was prepared from the corpuscles of oxalated horse blood, obtained through the kindness of the Mulford Biological Laboratories.

globin is much more soluble than oxyhaemoglobin. The osmometers consisted of collodion sacs attached to mercury manometers. The method of preparing the purified haemoglobin and the description of the osmometers are fully given in the previous communication [Wilson, 1923]. In the present experiments larger osmometers (holding about 15 cc. haemoglobin) were used in order to have enough haemoglobin solution for duplicate determinations of the hydrogen-ion concentration.

A typical experiment was carried out as follows. Several collodion sacs were filled with a freshly prepared 1 to 2 % solution of crystalline oxyhaemoglobin and dialysed against distilled water previously boiled and saturated with oxygen. The osmometer flask was loosely stoppered, so that sufficient carbon dioxide entered to make the outer fluid somewhat more acid than p_H 7·0. Most of the osmometers were kept in the ice box at about 2°. The outer fluid was renewed several times daily until the reaction remained constant and the osmotic pressure of the haemoglobin solution did not change. The osmotic pressure of a 1 % solution was about 10 mm. Hg, which agrees quite well with the calculated pressure using 16,600, the commonly accepted molecular weight of haemoglobin, as determined by several methods [Gamgee, 1898; Hüfner and Gansser, 1907; Barcroft and Hill, 1910].

Replacing the distilled water with weak acetic acid of p_H 3·8 to 4·0 (about 0·001 *M*) resulted in a four- to six-fold increase in the osmotic pressure. The acetic acid was renewed once or twice a day until the reaction of the outer fluid (tested colorimetrically) remained constant (one to two days). The elimination of the inner core of liquid in the osmometer by means of a glass float permitted the system to come to equilibrium rapidly. To be certain that equilibrium was obtained, however, most of the osmometers were allowed to stand for 3 or 4 days. The reactions of the haemoglobin solution and the acetic acid outer fluid were then determined by means of the hydrogen electrode. As a check on the permeability of the sacs to acetic acid, a sac containing distilled water (p_H 6·0–7·0) was dialysed against acetic acid of p_H 4·0. The p_H of the fluid inside the sac became 4·0 in less than 24 hours.

At the end of an experiment the haemoglobin from one or two of the osmometers was filtered and the percentage haemoglobin determined by evaporating a sample to dryness. It was always found that during the experiment some of the haemoglobin had been precipitated out of the solution, the reduction in concentration being 15 % or less. With the weak solutions used, this decrease in concentration introduced only a slight error in the comparison of osmotic pressure readings at the beginning and end of an experiment.

No attempt was made to determine the amount of conversion of oxyhaemoglobin into reduced haemoglobin and methaemoglobin. In the previous paper several reasons were given for believing that, under the conditions of these experiments, splitting of the haemoglobin molecule did not occur.

Typical results of the acetic acid experiments in four osmometers (two different samples of oxyhaemoglobin being used) are as follows:

Concentration of Hb solution %	Osmotic pressure of Hb solution: Against water mm. Hg	Osmotic pressure of Hb solution: Against acetic acid mm. Hg	p_H of acetic acid (outer fluid) at end of experiment	p_H of Hb (inner fluid): Found at end of experiment	p_H of Hb (inner fluid): Calculated on basis of Donnan equation
1·7	16	70	3·8	5·2	5·1
1·7	15	71	3·9	5·2	5·4
1·2	10	50	4·0	5·5	5·3
1·2	8	50	4·0	5·6	5·5

It will be seen from an examination of the fourth and fifth columns of the table that, at the end of such an experiment as has been described, the haemoglobin solution was considerably less acid than the acetic acid outer fluid, at a time when the osmotic pressure had increased four- to six-fold. This increased pressure might conceivably be due to one of a number of different causes, but, as was shown in the previous paper [Wilson, 1923], in every case, except that of the formation of haemoglobin acetate, the acetic acid would distribute itself so as to give the same hydrogen-ion concentration on each side of the membrane. In the case of the formation of haemoglobin acetate, however, the acetate ions therefrom would prevent an equal distribution of ions from the acetic acid, in accordance with Donnan's theory of membrane equilibrium. When the data are studied quantitatively, using the Donnan equation, it is found that the haemoglobin solution inside the osmometer should be less acid than the outer fluid, as was experimentally found to be the case. The increased osmotic pressure would be due to the haemoglobin ions and the acetate ions of the haemoglobin acetate (three to five acetate ions being necessary to account for the four- to six-fold increase in osmotic pressure). The counter osmotic pressure in these experiments is too slight to influence the calculations.

The calculations are necessarily rough, due to certain shortcomings in the data. The osmotic pressure readings are not quite as uniform as one would wish and the concentration of the haemoglobin undergoes a certain diminution during the course of the experiment. In postulating three to five acetate radicles for each haemoglobin radicle to account for the osmotic pressure change, it is assumed that there is no aggregation of the haemoglobin. If there were aggregation the ratio of acetate radicles to haemoglobin radicles would have to be larger.

Despite these sources of error, columns 5 and 6 in the table show that there is a relatively close agreement between the calculated and experimental values.

The calculations for the first of the experiments listed in the table above are given herewith.

Letting R be the cation of haemoglobin acetate, (1) outer fluid, (2) inner fluid, and x, y and z ion concentrations, we have the following scheme when equilibrium in the osmometer is attained.

$$\begin{array}{llll} & (2) & & (1) \\ (R) & (CH_3CO_2)\,z & & \\ z & & & \\ & (CH_3CO_2)\,z & & \\ & (CH_3CO_2)\,z & & \\ (H) & (CH_3CO_2) & (H) & (CH_3CO_2) \\ y & y & x & x \end{array}$$

$$(CH_3CO_2H)=(CH_3CO_2H)$$

$$y\,(y+3z)=x^2.$$

When the p_H of the outer fluid is 3·8, $x = 0{\cdot}00016\ N$. A 1·7 %haemoglobin solution (molecular weight of haemoglobin 16,600) is 0·0010 M, hence

$$z = 0{\cdot}0010\ M.$$

Substituting in $y\,(y + 3z) = x^2$,

$$y\,(y + 0{\cdot}003) = 0{\cdot}0000000256,$$

$$y^2 + 0{\cdot}003y = 0{\cdot}0000000256.$$

y^2, being infinitesimal as compared with the other values, may be disregarded. Hence

$$y = 0{\cdot}0000085.$$

The hydrogen-ion concentration (y) of the inner fluid which contains the haemoglobin acetate is, therefore, 0·0000085 N ($p_H = 5{\cdot}1$), while the hydrogen-ion concentration (x) of the outer fluid which contains only acetic acid is 0·00016 N ($p_H = 3{\cdot}8$). The experimental value, as seen in the table, is 5·2, instead of the calculated 5·1.

The results strongly indicate that the Donnan equilibrium was operating in these experiments and that haemoglobin acetate is formed when acetic acid is used as outer fluid in an osmometer with haemoglobin on the inside.

This conclusion can doubtless be extended to the formation of other acid salts of haemoglobin, under similar conditions, the hydrogen-ion concentration apparently being the determining factor. In the previous paper carbonic and lactic acids of the same p_H as the acetic acid used in these experiments gave identical osmotic pressure results.

Summary.

When haemoglobin is dialysed in an osmometer against acetic acid, there results a four- to six-fold increase in osmotic pressure above the value against distilled water. When equilibrium is reached the hydrogen-ion concentration of the haemoglobin solution is found to be considerably less than that of the acetic acid outer fluid. Such an unequal distribution of hydrogen ions may be explained by Donnan's theory of membrane equilibrium. The existence of a Donnan equilibrium under these conditions proves that haemoglobin acetate has been formed. The formation of haemoglobin acetate adequately explains the increased osmotic pressure.

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XIII. THE TYROSINASE-TYROSINE REACTION. II.

THE THEORY OF DEAMINATION.

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(*Received December 30th, 1924.*)

The action of tyrosinase on tyrosine by which the latter is converted into pigmented substances has been studied by many, and various theories have been evolved to explain the mechanism of the oxidation processes. All these theories, however, are based on the assumption that deamination of the tyrosine takes place. Chodat and Schweizer [1913] investigated the action of tyrosinase on glycine and stated that among the end-products were formaldehyde, CO_2 and NH_3. Bach [1914] applied this idea of deamination of the amino-acid to the action of the enzyme or mixture of enzymes on tyrosine and suggested that the first action of tyrosinase on tyrosine could be represented as

$$\underset{\text{Tyrosine}}{OH \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2) \cdot COOH} + H_2O + \underset{\substack{\text{Hydrogen}\\ \text{acceptor}}}{A} \rightarrow \underset{p\text{-Hydroxyphenylacetaldehyde}}{OH \cdot C_6H_4 \cdot CH_2 \cdot CHO} + NH_3 + CO_2 + AH_2$$

This reaction Bach attributed to an amino-acidase which splits up the tyrosine molecule into substances which are easily oxidised by the other enzyme component of tyrosinase, the phenolase (or peroxidase + H_2O_2). This action of the amino-acidase may be represented according to Bach as analogous to Strecker's reaction and results in deamination accompanied by or followed by decarboxylation.

$$\underset{\text{Tyrosine}}{OH \cdot C_6H_4 \cdot CH_2 \cdot CH(NH_2) \cdot COOH} + O\ (\text{active}) \rightarrow \underset{p\text{-Hydroxyphenylpyruvic acid}}{OH \cdot C_6H_4 \cdot CH_2 \cdot CO \cdot COOH} + NH_3 \quad ...(1)$$

$$OH \cdot C_6H_4 \cdot CH_2 \cdot CO \cdot COOH + (\text{Carboxylase}) \rightarrow \underset{p\text{-Hydroxyphenylacetaldehyde}}{OH \cdot C_6H_4 \cdot CH_2 \cdot CHO} + CO_2 \quad .....(2)$$

Similar views have been expressed by other workers. Folpmers [1916] carried out experiments which he interpreted as indicating that tyrosinase consists of two enzymes, a deaminising enzyme which converts tyrosine into *p*-hydroxyphenylacetaldehyde and a second enzyme which causes the further oxidation of the aldehyde (or its condensation product with ammonia) to form the black pigment melanin. Onslow [1923] accepts the view of Bach that the first step is the deamination of the tyrosine, followed by the further oxidation of these products and condensation with NH_3 to form melanin, and suggests that the reactions proceed as in equations (1) and (2). Haehn [1919] considers that tyrosinase consists of at least three different enzymes, one of which is a deaminase. Keeble, Armstrong and Jones [1913] have used the deamination theory in explaining pigment formation in plants. Abderhalden and Sickel

[1923] consider that the first stage in the tyrosinase-tyrosine reaction is a hydrolytic deamination accompanied by loss of CO_2. Thus all these theories involve the preliminary deamination of the tyrosine to form products which are subsequently oxidised (in some cases with condensation with NH_3) to form melanin. The experimental evidence supporting this view of deamination does not appear to be very weighty and the theory rests on a few isolated observations. Chodat and Schweizer [1913] identified H.CHO and NH_3 as products of the action of tyrosinase on glycine, and state that benzaldehyde is formed from phenylglycine, and acetaldehyde from alanine by the action of tyrosinase. These experiments have been repeated by Happold and Raper with negative results [1925]. Bach [1914] to support his theory of deamination attempted to show that *p*-hydroxyphenylacetaldehyde in the presence of the requisite amount of ammonia is oxidised by tyrosinase to the same end-products as those obtained in the tyrosinase-tyrosine reaction. In view of the difficulty in obtaining this aldehyde, Bach substituted the next lower member of this series, *p*-hydroxybenzaldehyde and claimed that by the action of peroxidase and H_2O_2 on this aldehyde in the presence of ammonia, colour changes similar to those of the tyrosinase-tyrosine reaction were obtained. These results, the rapid reddening of the solution followed by darkening to a violet-black colour, were not obtained when a phenolase was substituted for the peroxidase and H_2O_2 system, and this is attributed to the greater inhibitory action of NH_3 on phenolase. The only other evidence in support of the deamination theory appears to be that offered by Folpmers [1916], who isolated the *p*-nitrophenylhydrazone of benzaldehyde from a reacting mixture containing phenylglycine and plant juices, and an analogous hydrazone in extremely small quantities (too small for identification) when tyrosine was used.

The tyrosinase-tyrosine reaction may be represented as taking place in the following manner [Raper and Wormall, 1923]:

Tyrosine + (Tyrosinase) + O → Red pigment ... ... (3)

Red pigment → Colourless substance (probably an intramolecular change—spontaneous but greatly accelerated by warming) ... ... (4)

Colourless substance + O → melanin (accelerated by the phenolase of tyrosinase) (5)

If the reaction is carried out in slightly acid solution (p_H 6·0), the red substance is the main pigmented product formed during the first 6 or 8 hours, during which time from 50 % to 75 % of the tyrosine may be oxidised. This red substance is the first visible sign of oxidation although it may not be the first intermediate product in the oxidation process. On the assumption that deamination takes place, according to equation (1), the oxidation of tyrosine by tyrosinase will result in the liberation of an equivalent amount of NH_3 which should pass into solution. If no increase in the ammonia content of the reacting mixture takes place as the reaction proceeds, such a result would admit of two possibilities: (*a*) that no deamination of tyrosine occurs or (*b*) that deamination takes place but is immediately followed by the recombination of the NH_3 with the products of deamination (and decarboxylation) to form

a substance which in this case must be the red pigment. The second conjecture appears improbable and would involve the assumption that the deaminised products in the presence of NH_3 are rapidly converted by tyrosinase into the red pigment. That this assumption is incorrect will be shown later and thus the non-liberation of NH_3 as the reaction proceeds is not in favour of the theory of deamination.

In the present work, tyrosine solutions buffered at p_H 6·0 were acted upon by tyrosinase from potatoes and the ammonia content of the reacting mixture determined at intervals during the oxidation of at least two-thirds of the tyrosine present. No increase in the ammonia content of these solutions took place at any time, although the amount of NH_3 which should have been liberated according to equation (1) is quite appreciable. Additions to special samples of the solution of one-quarter of the amount of NH_3 which should theoretically be produced, calculated from the amount of tyrosine oxidised, resulted in these added quantities being estimated almost quantitatively, and thus satisfactory conditions appear to have been obtained. These ammonia estimations were carried out by the permutite filtration method [Whitehorn, 1923] for this method is of special application to slightly acid solutions. The ordinary methods for estimating NH_3 involve adding an excess of alkali and driving off the NH_3 into an acid solution and could not be applied in this instance, as the addition of the alkali would result in the rapid blackening of the solution. Thus the objection could then have been made that the free NH_3 in the solution had condensed after the addition of the alkali, with the products of tyrosine deamination, to form melanin. This inability to obtain any increase in the ammonia content of the reacting tyrosinase-tyrosine solution is strong evidence against the theory of deamination.

The property of permutite in removing all ammonia from neutral or slightly acid solutions may be utilised in obtaining qualitative evidence against the theory of deamination. The view that the final product, melanin, contains nitrogen is generally accepted and has been confirmed in the present work, but according to Bach [1914] this product is formed as the result of oxidation of the deaminised tyrosine accompanied by condensation with NH_3. If this is true, the removal of NH_3 from the reacting mixture should prevent the darkening of the solution, *i.e.* formation of melanin. It has been found however that no diminution in the production of melanin can be effected by shaking up the following solutions with permutite or filtration of these solutions through a layer of permutite: (1) the tyrosinase-tyrosine reaction mixture, (2) the clear red solution free from enzyme and (3) the colourless solution free from enzyme. Thus the removal of all the ammonia from these solutions does not inhibit the production of the final product melanin. It thus appears unlikely that ammonia is split off at any stage in the reaction.

From equation (1) it will be seen that *p*-hydroxyphenylpyruvic acid will be the first product or first non-nitrogenous product of the deamination of tyrosine and for this theory to hold good it is necessary that tyrosinase should

act on *p*-hydroxyphenylpyruvic acid in the presence of the requisite amount of ammonia to form the same end-products as the tyrosinase-tyrosine reaction. This acid has been prepared and it has been found that tyrosinase has no apparent action on it alone or with the addition of the calculated amount of NH_3. In addition, no increase in the amount of red pigment, colourless substance or melanin could be produced by adding this acid, with or without the addition of the calculated quantity of NH_3, to a reacting tyrosinase-tyrosine solution. This inability of tyrosinase to oxidise one of the intermediate products of the reaction according to the deamination theory, appears to be conclusive evidence against this theory.

The final product of the tyrosinase-tyrosine reaction, melanin, can be obtained in a comparatively pure state by the method described below, and prepared in this way, it is found to be a nitrogenous substance with a somewhat higher percentage of nitrogen than tyrosine. From the facts mentioned above, (*a*) the non-liberation of ammonia during the reaction, (*b*) the non-effect of removing any free ammonia from the reacting mixture on the production of red substance, colourless substance or melanin, and (*c*) the inability of tyrosinase to oxidise *p*-hydroxyphenylpyruvic acid in the presence of NH_3, the conclusion must be drawn that no deamination takes place in the tyrosinase-tyrosine reaction. Further, since the final product, melanin, contains approximately the same percentage of nitrogen as tyrosine and as the intermediate products have certain properties which lead to the supposition that they are nitrogenous, it is suggested that all the intermediate products and final product contain nitrogen and that at no stage of the reaction is nitrogen split off in the form of ammonia.

Experimental.

In all the experiments described below except where the contrary is stated, dialysed potato juice prepared in the manner previously described [Raper and Wormall, 1923] was used as the source of tyrosinase. All the reactions were carried out in phosphate buffered solutions since the p_H of the solution has a very marked influence on the nature and quantity of the products obtained.

Ammonia content of the reacting tyrosine-tyrosinase mixture.

For this determination, the tyrosine solution was buffered at p_H 6·0, this reaction being considered most suitable for the purpose. In alkaline solutions and to a less extent at p_H 7·0 the red substance is rapidly converted into melanin, and according to the theory of Bach this melanin formation is due to the recombination of the deaminised fraction with ammonia and to condensation processes. Thus the best conditions for the detection and estimation of the liberated ammonia will be those in which the amount of melanin formed is at a minimum, while the amount of tyrosine oxidised is at or near the maximum. These conditions are best satisfied by allowing the enzyme action

to proceed in slightly acid solutions, and at p_H 6·0 the velocity of the oxidation is not greatly inferior to that at p_H 7·0 or 8·0 [Raper and Wormall, 1923].

Dialysed potato juice was added to the tyrosine solution, toluene added and a constant current of air drawn through this and a control solution containing no tyrosine, as previously described for the determination of the velocity coefficients. Four samples of 60 cc. each were withdrawn from each of the two solutions and half of these samples were heated to convert the red pigment where present into the colourless substance. The ammonia content of all these samples was then determined by the method of Whitehorn [1923], which involves the filtration of the solution through a layer of permutite, the liberation of the NH_3 by the addition of alkali to the permutite and nesslerisation. No increase in the ammonia present in the reacting tyrosine-tyrosinase solution could be observed at any time and since about two-thirds of the tyrosine present was oxidised during the time of observation, the amount of NH_3 liberated according to the deamination theory would have been appreciable.

Method of ammonia estimation.

Each sample (60 cc.) was filtered slowly through about 5 cc. of finely granular permutite contained in a $CaCl_2$ tube, the permutite having previously been washed with 2 % acetic acid to neutralise any alkali present and several times with distilled water. When the solution whose ammonia content was to be determined had filtered through, the permutite was washed five times with 10–20 cc. of distilled water and finally rinsed with the minimum quantity of water into a 100 cc. graduated flask. 5 cc. of 10 % NaOH were added to each suspension of permutite containing ammonia, the solution shaken for a minute and 15 cc. of modified Nessler's solution (Bock and Benedict formula) added. The solutions were allowed to stand for 15 minutes, centrifuged when necessary and estimated colorimetrically against a standard prepared as follows: 2 cc. of a standard NH_4Cl solution (5 cc. contain 1 mg. NH_3) + 20 cc. dist. H_2O + 5 cc. 10 % NaOH + 15 cc. mod. Nessler's solution. The solution was shaken, allowed to stand for 15 minutes and then made up to 100 cc.

Duplicate estimations were carried out after each interval of time and "test estimations" were also made by adding to special samples of the reaction mixture 2 cc. of the standard NH_4Cl solution (2 cc. contain 0·40 mg. NH_3). The procedure for these test estimations was the same as that for the ordinary samples, and these added amounts of NH_3 were estimated almost quantitatively.

Tyrosine estimations.

These were carried out on 20 cc. samples withdrawn from the solutions at the commencement of the reaction and after 7½ hours and the method used was the bromination method previously described. The results show that approximately two-thirds of the tyrosine was oxidised during the experiment.

Solution A. 1000 cc. phosphate buffered 0·05 % tyrosine solution (p_H 6·0) + 100 cc. dial. potato juice + 30 cc. toluene.

Solution B. 1000 cc. phosphate buffered water (p_H 6·0) + 100 cc. dial. potato juice + 30 cc. toluene.

Solution A (red) refers to the samples of the reacting mixture which were used unchanged for the estimations.

Solution A (colourless) refers to samples which had been boiled to convert the red pigment into the colourless substance. This conversion was brought about by adding to the sample 1 cc. $N/10$ HCl, boiling, filtering and finally adding 1 cc. $N/10$ NaOH.

Results. Ammonia content of the samples in mg. per 60 cc.

Solution A (red)	0·16	0·17	0·17	0·16
Solution A (colourless) ...	0·17	0·18	0·20	0·18
Solution B (unboiled) ...	0·15	0·17	0·16	0·17
Solution B (boiled) ...	0·15	0·18	0·16	0·16
Tyrosine in Solution A (%)	0·045	—	—	0·013
Time (hrs)	0	3	5	7½

The amount of NH_3 which would have been liberated by the deamination of the tyrosine which was oxidised in 7½ hours is 1·80 mg. per 60 cc., and as less than a quarter of this amount, if added to a sample, was estimated in full, it appears inconceivable that deamination or the liberation of ammonia takes place during the action of tyrosinase on tyrosine.

The action of tyrosinase on p-*hydroxyphenylpyruvic acid.*

This acid has been prepared by the method used by Neubauer [1909], but the filtrate from the benzoic acid was immediately extracted with successive quantities of ether until almost all of the keto acid had been extracted, as shown by the inability of the neutralised aqueous solution to give more than a faint green colour with dil. ferric chloride solution. The ethereal solution containing the *p*-hydroxyphenylpyruvic acid was distilled under reduced pressure to remove the solvent, the residue dissolved in the minimum quantity of hot alcohol and poured into cold water. A white crystalline ppt. of the keto acid separated, was filtered off, washed with a little benzene and dried *in vacuo*. In some cases the acid was practically pure but in other cases had to be recrystallised from hot water (M.P. 220°). A further quantity of the acid can be recovered from the mother liquor. Solutions containing 0·05 % of this keto acid were then prepared and buffered at p_H 6·0, 7·0 and 8·0. These solutions were then acted upon by potato juice both with and without the amount of NH_3 (as NH_4Cl) theoretically required.

Controls were also set up at each p_H, using only the phosphate buffers and potato juice. The whole of the experiment was then repeated using dialysed potato juice as the source of tyrosinase and the results were exactly the same as those quoted below.

Experiments were then carried out to see whether the addition of *p*-hydroxyphenylpyruvic acid together with the theoretical amount of NH_3 to

reacting tyrosine-tyrosinase solutions would increase the yield of red pigment, colourless substance or melanin. No increase in the amount of either of the intermediate compounds or of the final product took place.

Observations.

Solutions used: I. 0·05 % phosphate buffered tyrosine solution (p_H 6·0, 7·0 and 8·0).
II. 0·05 % phosphate buffered *p*-hydroxyphenylpyruvic acid solutions (p_H 6·0, 7·0 and 8·0).
1·5 % NH_4Cl solution. Fresh and dialysed potato juice.

			1 hour	4½ hours	36 hours
1.	100 cc. Solution I (p_H 6·0)	+10 cc. potato juice	Red	Red	Black
2.	100 „ „ II (p_H 6·0)	+10 „ „ „	No change	No change	No change
3.	100 „ „ II (p_H 6·0)	+10 „ „ „ +1 cc. 1·5 % NH_4Cl	No change	No change	No change
4.	100 „ „ I (p_H 7·0)	+10 cc. potato juice	Red	Black-red	Black
5.	100 „ „ II (p_H 7·0)	+10 „ „ „	No change	No change	No change
6.	100 „ „ II (p_H 7·0)	+10 „ „ „ +1 cc. 1·5 % NH_4Cl	No change	No change	No change
7.	100 „ „ I (p_H 8·0)	+10 cc. potato juice	Red-black	Black	Black
8.	100 „ „ II (p_H 8·0)	+10 „ „ „	No change	No change	No change
9.	100 „ „ II (p_H 8·0)	+10 „ „ „ +1 cc. 1·5 % NH_4Cl	No change	No change	No change

These results lead to the conclusion that *p*-hydroxyphenylpyruvic acid is not an intermediate product in the oxidation of tyrosine by tyrosinase and thus the view that deamination occurs according to equations (1) and (2) appears to be untenable.

The preparation and nitrogen content of the melanin.

2 g. of tyrosine were dissolved in 4 litres of water, 400 cc. of fresh potato juice added and the reaction of the solution adjusted to p_H 6·0. The solution was then warmed to 37° and stirred vigorously in a wide vessel. When the intensity of the red colour reached a maximum and the solution was beginning to darken slightly, usually after 5–6 hours, the stirring was stopped. The solution was transferred to a 5-litre flask, 40 cc. of 10 % acetic acid added and the liquid boiled as quickly as possible. After being thoroughly cooled, the solution containing the coagulated proteins was filtered, and the filtrate allowed to stand overnight after being made definitely alkaline with 100 cc. 10 % Na_2CO_3. On the addition of the alkali the colourless solution rapidly darkened and the formation of the melanin was accelerated by shaking with air. The melanin obtained in this way could not be filtered off, but was obtained in the precipitated condition by adding 200 cc. 10 % acetic acid and boiling the solution. The melanin was filtered off, leaving a clear solution and was purified as far as possible by boiling with dil. HCl, then with successive quantities of water, and washing with hot water. It was dried on porous plates *in vacuo* over H_2SO_4. Examined under the microscope it appeared to be amorphous and was hard enough to be powdered finely. Nitrogen was present in considerable quantity according to the qualitative test (fusion with Na, etc.), but no sulphur. The absence of sulphur is evidence in favour of the view that the melanin is free from nitrogenous substances derived from the

potato juice, for the proteins of this juice have been found to contain sulphur. Four different samples of melanin were prepared by the above method, the yield being 0·5 g. to 0·8 g. melanin from 2 g. tyrosine, and the nitrogen content determined by the Kjeldahl method. The results showed that 8·43 %, 8·30 % and 8·47 % of nitrogen were present in three of the preparations by the Kjeldahl method and 8·65 % by the Dumas method. This is rather higher than the percentage of N in tyrosine (7·73 %), and is probably due to the fact that although oxygen has been absorbed by the tyrosine, molecules of water have been removed in the condensation processes, with resulting slight increase in the percentage of nitrogen present.

Summary and Conclusions.

1. Evidence against Bach's theory of deamination of the tyrosine has been obtained in the following manner and appears to be a conclusive refutation of this view.

(*a*) A reacting tyrosinase-tyrosine solution shows no increase in ammonia content during the oxidation of at least two-thirds of the tyrosine present.

(*b*) Removal of all the ammonia initially present in the solutions or formed by any part of the oxidation process does not affect in any way the formation of the intermediate products, *i.e.* the red and colourless substances, or the final product melanin.

(*c*) Tyrosinase has no action on solutions of *p*-hydroxyphenylpyruvic acid either with or without the necessary ammonia for recondensation.

2. The final product of the tyrosinase-tyrosine reaction, melanin, is a nitrogenous compound with a slightly higher percentage of nitrogen than tyrosine (8·40 % against 7·73 % N in tyrosine).

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XIV. THE TYROSINASE-TYROSINE REACTION. III.

THE SUPPOSED DEAMINISING ACTION OF TYROSINASE ON AMINO ACIDS.

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For some years it has been generally accepted that the enzyme tyrosinase will bring about the oxidation of amino acids with the production of ammonia. This view was first suggested by Chodat and Schweizer [1913] to explain the production of formaldehyde and ammonia from glycine when it was submitted to the action of tyrosinase from potatoes. The process of deamination has been made the basis of a theory as to the components of the enzyme tyrosinase by Bach [1914], who accepts the view that tyrosinase has a deaminising action on tyrosine. Folpmers [1916] isolated a small amount of benzaldehyde as the *p*-nitrophenylhydrazone from the products of the action of tyrosinase on phenylaminoacetic acid, thus supporting the deamination hypothesis.

A study of the experimental evidence on which the deamination theory was based led us to the opinion, that certain of the experiments should be repeated and extended, and the results of this work are included in the present paper.

The tyrosinase used throughout this investigation was obtained by the dialysis of potato juice through moderately permeable collodion sacs. An attempt was made in the first instance to obtain from alanine, by the action of tyrosinase, the aldehyde corresponding to it, namely, acetaldehyde. This proved abortive although the enzyme preparations used were very active as shown by their action on tyrosine. It was then found that under the conditions of the experiments, in which free access of oxygen is a necessity, small traces of added acetaldehyde disappeared, probably by oxidation. Attempts to detect formaldehyde and benzaldehyde as products of the action of tyrosinase on glycine and phenylaminoacetic acid respectively were also negative. The search for aldehydes as products of the tyrosinase reaction was therefore discontinued and instead experiments were carried out in which the ammonia, if any, produced by the action of tyrosinase on amino acids could be estimated. The acids chosen were glycine, alanine, phenylaminoacetic, phenylalanine and tyrosine. The conditions were such that had any ammonia been produced it would have been estimated easily. These experiments also provided no evidence of deamination and in this respect agreed with the experiments of Raper and Wormall on tyrosine [1925] carried out by other methods.

Bach [1914] has suggested that after deamination the ammonia recombines with some other product of the enzyme reaction—possibly the aldehyde—and as this might conceivably lead to the non-detection of ammonia as a product of the reaction, a series of experiments was carried out to determine whether any change in the amino N content of the system took place when tyrosinase acted on glycine, alanine or phenylaminoacetic acid. The results were completely negative. In addition, measurements have been made of the oxygen taken up when tyrosinase acts on tyrosine, glycine and alanine under identical conditions. A considerable oxygen absorption was observed with tyrosine but none with the other two amino acids. We have thus come to the conclusion that potato tyrosinase, under the conditions in which the enzyme is very active, has no deaminising action on amino acids.

These results necessitated a re-examination of the experiments of Chodat and Schweizer in which they claimed to have obtained evidence of deamination of glycine by potato tyrosinase, by the detection of formaldehyde and ammonia as products of the reaction. On reading over the records of Chodat and Schweizer's experiments, it was noticeable that success was only achieved when *p*-cresol was added to the reacting system. This addition was made, according to Chodat and Schweizer, in order to prevent interaction of the ammonia and formaldehyde produced by the deamination process, since the uncertainty in detecting them when *p*-cresol was not present was attributed to their further reaction with each other.

On repetition of these experiments we have been able to confirm that when *p*-cresol is present along with glycine and tyrosinase, ammonia is liberated and there is a diminution in the amino-nitrogen content of the system.

The evidence in favour of formaldehyde production adduced by Chodat and Schweizer we have not been able to confirm. *p*-Cresol itself reduces ammoniacal silver nitrate solution so that in its presence a reduction of this reagent cannot be accepted as evidence of the presence of formaldehyde, a point seemingly overlooked by Chodat and Schweizer.

Schryver's reaction for formaldehyde—the second reaction which they used—has failed in our hands to show the presence of formaldehyde when tyrosinase acts on glycine. This reaction will detect with ease 1 part in 10,000 of formaldehyde so that if it is produced even in traces it should have been detected. We are led therefore to the conclusion that the deamination found by Chodat and Schweizer was due to the presence of *p*-cresol and tyrosinase and not to tyrosinase alone, and as Chodat has shown that the latter attacks the *p*-cresol, it is probable that the deamination is produced by some oxidation product of *p*-cresol acting on the amino acid.

It was of interest to compare the action of other phenolic and related substances with that of *p*-cresol, and this has been done with phenol, resorcinol, quinol, catechol, *p*-benzoquinone and *o*-benzoquinone.

Only those phenols which are likely to give an *ortho*-dihydric-phenol or *ortho*-quinone derivative on oxidation gave positive results in the deamination

experiments. Under the conditions in which the experiments were carried out, even in the absence of tyrosinase, *o*-quinone produced deamination. This suggests that the phenols which were found to be active display this activity because they yield *o*-quinone or a derivative of it (*e.g.* 3·4 toluquinone from *p*-cresol), on oxidation by tyrosinase and that this then reacts with the amino acid.

It should be noted that M. W. Onslow [1923] has suggested that tyrosinase is a mixture of enzymes; a water splitting enzyme (reductase or deaminase), a carboxylase and an oxydase (consisting of an oxygenase, a dihydroxy substance and a peroxydase), and that she suggests that *p*-cresol is oxidised by traces of active oxygen to 3·4-dihydroxytoluene, but there is no assumption of the formation of an *o*-quinone which reacts with the amino acid and causes deamination.

The observations recorded in this paper indicate that tyrosine is acted on by tyrosinase, not because it is an amino acid but because it possesses a phenolic hydroxyl group. Phenylalanine, which only differs from tyrosine in this particular, is not oxidised by tyrosinase. It seems likely therefore that a preliminary stage in the oxidation of tyrosine by tyrosinase may be the formation of an *o*-quinone derivative which then undergoes an intramolecular reaction with the side chain containing the amino group, or reacts with another molecule of tyrosine. The amino group is, however, not split off as ammonia, as it is when *o*-quinone attacks other amino acids.

Experimental.

In the experiments with tyrosinase described in this section, a phosphate buffer mixture adjusted to p_H 7 was used and toluene was added as an antiseptic.

I. *The Alleged Aldehyde Formation due to the Action of Tyrosinase on Amino Acids.*

(*a*) *From alanine.* For the detection of acetaldehyde, *p*-nitrophenylhydrazine was used in acetic acid solution. Two large flasks *A* and *B* were taken and into each was placed a mixture of 100 cc. dialysed potato juice, 100 cc. of the buffer solution and 10 cc. of toluene, in addition 1 g. of alanine was placed in *A*. The two flasks were left at room temperature for 24 hours, after which the contents were made just acid to Congo red by the addition of phosphoric acid and distilled.

The distillate was first caused to pass through a percolator packed with broken ice, after which it was collected in two separate portions, 25 cc. in volume, in a receiver, surrounded by melting ice, which contained the maximum theoretical amount of a *p*-nitrophenylhydrazine solution necessary for the conversion of any aldehyde formed into the *p*-nitrophenylhydrazone. A very faint turbidity was produced by the distillate obtained from *A*, while that from *B* produced no change.

This failure to detect acetaldehyde in the products of the reaction led to the consideration of the possibility under the conditions of the experiment of

(1) condensation of the aldehyde with itself,
(2) loss of aldehyde by oxidation and
(3) a loss of aldehyde by volatilisation.

To test these possibilities the following experiments were undertaken:

(1) The above experiment was repeated with the phosphate buffer adjusted to p_H 6·5 so as to prevent the aldol condensation of acetaldehyde. Again no acetaldehyde was detected as a reaction product, thus ruling out autocondensation as an explanation of its possible disappearance from the system.

(2) Two 2-litre flasks were taken and into each was placed a mixture containing 50 cc. dialysed potato juice, 50 cc. buffer solution and 0·05 cc. acetaldehyde; the one was stoppered and the other unstoppered. Two similar flasks were set up at the same time as controls, water displacing the enzyme preparations previously used. After standing for 24 hours each flask was tested for acetaldehyde by the *p*-nitrophenylhydrazine reaction as previously described. There was a greater amount of hydrazone formation in the experiments in which water had taken the place of the enzyme and also in the case of the stoppered flasks as compared with the unstoppered; the greatest difference was, however, *small* and it was concluded from the results that while the loss of acetaldehyde due to possible oxidation was by no means negligible, that due to evaporation could be ignored.

(*b*) *From glycine.* With a view to testing whether formaldehyde was produced from glycine by the action of tyrosinase, 0·25 g. of glycine dissolved in 50 cc. of buffer solution was mixed with 50 cc. of dialysed potato juice and 10 cc. of toluene and exposed to the air in a 2-litre flask for 24 hours side by side with a control which contained no glycine. At the end of the period portions tested with Schryver's reagent gave no formaldehyde reaction and no formaldehyde could be detected in the distillate collected over ice in the manner previously described for acetaldehyde.

(*c*) *From phenylaminoacetic acid.* Folpmers' experiment on the formation of benzaldehyde has also been carefully repeated with negative results. 0·2 g. of phenylaminoacetic acid was placed in a mixture of 25 cc. dialysed potato juice, 25 cc. buffer solution and 10 cc. toluene; a similar control with water replacing the enzyme was set up. After 24 hours' exposure to the air no benzaldehyde formation could be demonstrated in either case. The phenylaminoacetic acid used in this experiment was prepared in the usual way by the hydrolysis of its nitrile hydrochloride and was carefully purified by recrystallisation from dilute acetic acid. It seemed possible that a contamination of the phenylaminoacetic acid with its nitrile as a result of incomplete hydrolysis might have accounted for Folpmers' results, so to test this 1 g. of the nitrile hydrochloride was subjected to the action of tyrosinase for 24 hours in the presence of air and the solution subsequently acidified and distilled.

From the distillate 0·42 g. of the *p*-nitrophenylhydrazone of benzaldehyde was obtained which was practically pure after one crystallisation. It appears therefore that the apparent deamination of phenylaminoacetic acid demonstrated by Folpmers may possibly have been due to the contamination of the phenylaminoacetic acid with its nitrile. In his paper, however, Folpmers does not state the source of the phenylaminoacetic acid which he used.

II. *The Alleged Ammonia Formation.*

The method of detecting ammonia formation was based on Folin's method of estimating ammonia in urine. The apparatus, which was in duplicate, consisted of three bottles, *A*, *B* and *C*, which were connected in series and air was aspirated through both sets by the same pump; *A* contained 20 % H_2SO_4 so that any air drawn through it would be ammonia free, *B* was the reaction bottle in which the enzyme-buffer mixture was placed and *C* the absorption bottle, contained 100 cc. of *N*/50 H_2SO_4.

The whole having been connected up, 10 cc. of toluene, 25 cc. of dialysed potato juice and 25 cc. of the buffer solution containing a definite amount of the amino acid were added to the reaction bottle *B*; a control mixture without the amino acid being placed in the reaction bottle of the duplicate series.

By means of a water pump the whole was aerated gently for 24 hours. At the end of this time about 4 g. of Na_2CO_3 and a few drops of capryl alcohol were added to each reaction bottle, and any free ammonia in the reaction bottle was drawn over into the respective absorption bottles.

The figures represent the cc. of *N*/10 NaOH used in the titration of the acid in the absorption bottles. Any formation of ammonia in the direct experiment would cause this figure to be lower than the figure from the control bottle. In no case was deamination observed.

	Experimental		Control	
	Readings	Average	Readings	Average
Alanine, 0·25 g.	19·90 19·94 19·95 19·95	19·93	20·01 19·99 19·95 19·99	19·98
Glycine. 0·5 g.	19·96 19·89	19·93	20·00 20·01	20·01
Phenylalanine, 0·25 g.	19·98 19·98	19·98	19·90 20·04	19·97
Tyrosine, sat. sol.	19·95 19·97	19·96	20·02 20·04	20·03
Phenylaminoacetic acid, sat. sol.	19·85 19·95	19·90	19·85 19·95	19·90

The small differences observed are within the experimental error.

Study of the Amino Nitrogen Content of the System during the Period of Aeration.

The reaction and control mixtures were aerated in the same apparatus as that used in the ammonia determination, samples were withdrawn at intervals from both mixtures and the amino nitrogen content of these estimated by means of the micro van Slyke method.

The amino acids studied were alanine, glycine and phenylaminoacetic acid; in the case of the first two, 0·5 cc. of the enzyme-buffer mixture was used for each determination, in the case of the last 3·5 cc. were taken. The figures given are in cc. of nitrogen at N.T.P. The amino acid was dissolved in a mixture of equal parts, enzyme and buffer solutions.

Amino acid	Time hours	Experiment cc.	Control cc.	Difference cc.
Alanine: 0·4645 g. in 50 cc. enzyme-buffer solution	0	1·33	0·26	1·07
	24	1·39	0·26	1·13
	30	1·41	0·29	1·12
Glycine: 0·45 g. in 60 cc. enzyme-buffer solution	0	1·50	0·36	1·14
	2	1·51	0·37	1·14
	26	1·55	0·41	1·14
Phenylaminoacetic: 0·0349 g. in 60 cc. enzyme-buffer solution	0	0·53	0·26	0·27
	24	0·62	0·35	0·27
	26	0·62	0·35	0·27

These experiments show that the amount of amino nitrogen in the system in each case was unchanged after 24 hours or more.

As a useful comparison, figures were obtained showing the amount of tyrosine oxidised under the above conditions. The method adopted for the estimation was the modification of Miller's method devised by Raper and Wormall [1923]. A mixture of 60 cc. of the buffer solution containing 0·0264 g. of tyrosine and 60 cc. of dialysed potato juice having been divided into two parts 10 cc. of toluene were added to each, both portions being then aerated as in previous experiments. After 24 hours 0·0146 g. tyrosine was left, so that 0·0118 g. of tyrosine out of a possible 0·0264 g., or 44·7 %, had been oxidised.

Oxygen Absorption in Tyrosinase-Amino-Acid Mixtures.

If the alleged deamination of amino acids by tyrosinase is carried out by the same chemical processes which are involved when this enzyme acts on tyrosine then oxygen should be absorbed as it certainly is when tyrosine is the substrate. To investigate this experiments have been carried out in a Barcroft apparatus. In one bottle a buffered solution of the amino acid was used and in the other the buffer solution only. Toluene was present in each bottle and 0·2 cc. of enzyme was placed in the cup of each stopper. When equilibrium had been obtained with the bottles open to the air, they were connected to the manometer and the enzyme solution spilt into the bottles. Experiments were carried out with tyrosine (saturated solution in the buffer mixture), glycine and alanine, the amino acids all being used in approximately equimolecular quantities. No oxygen absorption was observed in 6 hours with glycine or alanine. There was, in fact, a slight increase in the case of alanine (12 $mm.^3$). With tyrosine a considerable oxygen absorption occurred, which at the end of 18 hours measured 0·1053 cc. These experiments indicate therefore that no oxidation of glycine or alanine takes place under conditions in which a considerable oxidation of tyrosine can be demonstrated. They do not support the view that tyrosinase exerts an oxidative deamination on amino acids in general.

III. *The Effect of the Addition of Phenols and Related Substances to the System Tyrosinase-Amino-Acid.*

Preliminary experiments showed that deamination and a diminution in the amino N content of the system took place when tyrosinase acted upon glycine in the presence of *p*-cresol. A systematic study was therefore made of the influence of *p*-cresol on other amino acids in the presence of tyrosinase and the investigation was extended to include other phenols and allied substances. The phenols, etc., used were phenol, catechol, resorcinol, quinol and *p*-benzoquinone. Of these only *p*-cresol, phenol and catechol produced deamination. The results are tabulated below. The method of experiment was as follows. The amino acid was dissolved in 50 cc. of the phosphate buffer solution, 50 cc. of dialysed potato juice were added and the solution divided into two equal portions. To one 10 cc. of a solution of the phenol or related substance were added and to the other, which served as a control, 10 cc. of water. Toluene was added as an antiseptic. The amino N was determined in a small sample from each at the beginning of the experiment and usually after 24 and 48 hours' gentle aeration at room temperature. Finally, any ammonia produced was liberated by adding solid sodium carbonate and blowing it over into $N/50$ sulphuric acid. The titration figures given in the last column but one give the amount of $N/10$ NaOH used in the titration of the excess of acid not neutralised by ammonia.

Phenol etc. used	Amino acid used		Amino N in cc. 0 hr.	24 hr.	48 hr.	cc. $N/10$ NaOH	NH_3 liberated mg.	Deamination
p-Cresol, 0·4 %	Alanine, 0·5 g.	Exp.	2·36	2·08	1·72	18·10	4·5	Positive
		Cont.	2·34	—	2·33	20·75		
	Phenylalanine, 0·3 g.	Exp.	0·93	0·83	0·73	18·50	3·8	Positive
		Cont.	0·94	—	0·86	20·75		
	Leucine, 0·5 g.	Exp.	1·63	—	0·91	17·67	5·0	Positive
		Cont.	1·59	—	1·60	20·65		
Phenol, 1·0 %	Glycine, 0·4 g.	Exp.	2·27	1·81	1·33	15·28	8·8	Positive
		Cont.	2·22	—	2·21	20·48		
	Alanine, 0·5 g.	Exp.	2·26	1·85	—	18·65	3·0	Positive
		Cont.	2·29	2·29	—	20·42		
Resorcinol, 0·5 %	Glycine, 0·3 g.	Exp.	1·90	1·95	—	19·81	0	Negative
		Cont.	1·78	1·76	—	19·76		
	Alanine, 0·5 g.	Exp.	2·51	2·46	2·43	19·71	0	Negative
		Cont.	2·52	2·49	2·52	19·71		
Quinol, 1·0 %	Glycine, 0·45 g.	Exp.	2·46	2·49	—	19·86	0	Negative
		Cont.	2·45	2·41	—	19·71		
	Alanine, 0·55 g.	Exp.	2·82	—	2·82	19·61	0	Negative
		Cont.	2·85	2·83	2·80	19·61		
	Phenylalanine, 0·3 g.	Exp.	1·11	1·16	—	19·99	0·17*	Negative
		Cont.	1·04	1·10	—	20·09		
p-Benzoquinone, 1·0 %	Glycine, 0·25 g.	Exp.	1·62	1·59	—	19·48	0·1*	Negative
		Cont.	1·61	1·60	—	19·54		
	Alanine, 0·5 g.	Exp.	2·64	2·66	—	19·95	0·17*	Negative
		Cont.	2·64	2·67	—	20·05		
	Phenylalanine, 0·25 g.	Exp.	0·99	0·98	—	19·49	0	Negative
		Cont.	0·98	1·00	—	19·50		
Catechol, 0·5 %	Glycine, 0·25 g.	Exp.	1·84	1·73	—	18·36	2·4	Positive
		Cont.	1·85	1·87	—	19·78		
	Alanine, 0·55 g.	Exp.	2·84	2·37	—	18·28	2·3	Positive
		Cont.	2·84	2·83	—	19·62		

* These small amounts are within the experimental error.

Since only those phenols which were likely to give an *ortho*-quinone on oxidation caused deamination to take place, experiments were carried out with *o*-quinone itself and it was found that this substance attacked amino acids, even in the absence of tyrosinase, causing a liberation of ammonia and diminution in the amino N content of the reacting system. The *o*-quinone was prepared by Willstätter and Müller's method [1908]. The quinone is very unstable and two methods were used to demonstrate its action. In the first the quinone obtained from 0·25 g. catechol was thrown down from its solution in ether by the addition of light petroleum, washed with the latter solvent and then redissolved in ether. This solution was added to the solution of the amino acid (0·175 g. glycine in 60 cc. of the buffer mixture) and a similar solution to which no quinone was added served as a control. Aeration was carried out for 24 hours. The initial amino N figures were 2·01 and 1·99 cc. for the experiment and 2·02 and 1·99 for the control; the final figures were 1·91 and 1·90 cc. for the experiment and 2·00 and 1·99 cc. for the control. The *N*/10 NaOH used for the acid in the ammonia absorption bottle was 18·88 cc. for the experiment and 19·58 cc. for the control. Both these results therefore provided satisfactory evidence of deamination. In the second experiment, the quinone from 0·5 g. catechol was precipitated directly from the ether in the reaction bottle and was not further purified, hence it would contain a small amount of catechol. The amino acid solution was added directly to this and 0·25 g. catechol was added to the control solution in order to allow for any possible deamination due to catechol alone. An experiment with 0·2 g. of glycine gave the following results. Initial amino N figures: experiment 2·38 and 2·40 cc., control 2·43 and 2·45 cc.; final amino N: experiment 1·96 and 1·95 cc., control 2·49 and 2·51 cc. The titration figures were: experiment 15·32 cc., control 19·84 cc. An experiment with alanine (0·25 g.) carried out in the same way with the quinone from 0·25 g. catechol gave initial figures of 2·45 cc. and 2·44 for the experiment and control respectively and final figures of 2·285 cc. and 2·50 cc. The titration figures were: experiment 19·30 cc., control 20·00 cc.

The control solution containing catechol in these experiments coloured slightly to a faint pink during the 24 hours' aeration but it did not produce any deamination, consequently the assumption is justified that the observed deamination in the presence of catechol and *o*-quinone was due to the latter.

Summary and Conclusions.

1. No evidence of aldehyde formation, liberation of ammonia or a diminution in amino nitrogen has been found when potato tyrosinase acts upon glycine, alanine or phenylaminoacetic acid.

2. When tyrosinase acts upon tyrosine to form melanin or its precursors oxygen is absorbed, but no oxygen absorption occurs when equivalent molecular solutions of glycine and alanine are exposed to the action of the enzyme.

3. The ammonia formation observed by Chodat and Schweizer when tyrosinase acts upon amino acids is due to the *p*-cresol which was added to the reacting system. Phenol and catechol exert the same action but not resorcinol, quinol or *p*-benzoquinone.

4. *o*-Benzoquinone alone is capable of causing deamination of amino acids in the same way as the system *p*-cresol-tyrosinase.

5. It is suggested that the deamination which takes place when tyrosinase acts upon certain phenols in the presence of amino acids is due to the preliminary formation of an *ortho*-quinone derivative which then attacks the amino acid, with the liberation of ammonia and the formation of deeply pigmented substances (Chodat's *p*-cresol-tyrosinase reaction).

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XV. THE EFFECTS OF CALCIUM GLYCEROPHOSPHATE, SODIUM GLYCEROPHOSPHATE AND SODIUM DIHYDROGEN PHOSPHATE UPON THE SKELETON OF RATS KEPT ON A DIET DEFICIENT ONLY IN FAT-SOLUBLE FACTOR.

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I. Historical.

Mellanby [1920, 1921], Korenchevsky [1921, 1922], Goldblatt [1923], Zilva, Golding and Drummond [1924] have shown that a diet deficient in fat-soluble factor produces rickets in puppies, rats and pigs. An increase in the calcium content of such a diet does not cure this type of rickets [Korenchevsky, 1923].

It is also known that human rickets cannot be cured by an increased ingestion of calcium salts. In Orgler's opinion [1911] the capacity to deposit calcium in the bones is lost during the active stage of this disease.

Grosser [1920], however, found that marked calcium retention occurred in rickety children when this element was introduced subcutaneously in the form of a glycerophosphate. As far as we know, Grosser's observations remain unique and this question has not been studied clinically or experimentally since. However, they are not only theoretically important but may be of practical use in the treatment of diseases affecting the calcium metabolism.

II. Technique of the present investigations.

We conducted 19 experiments on 20 litters containing altogether 148 rats. Clearly, for the proposed experiments, only a diet containing an adequate amount of calcium and phosphorus could be used. Therefore, during the experiment, our animals were kept on a rickets-producing diet deficient only in fat-soluble factor, because other similar diets are either deficient in calcium or phosphorus. In order to discover whether the compound calcium glycerophosphate or the glycerophosphate radical alone is the influencing factor, we divided each of our litters into two or more of the following groups.

Group I. Control group of rats kept on a diet deficient in fat-soluble factor.

Group II. Rats on the same deficient diet, a solution of calcium glycerophosphate being injected subcutaneously.

Group III. Rats on the same diet, a solution of sodium glycerophosphate being injected subcutaneously.

Group IV. Rats on the same diet, a solution of sodium dihydrogen phosphate being injected subcutaneously.

Group V. Rats on the same diet, but to which has been added calcium glycerophosphate (to show the influence of the ingestion of calcium glycerophosphate).

Two diets, $-$ A and $-$ A_2, were used, both being deficient only in the fat-soluble factor; the composition of these diets is given in our previous papers [Korenchevsky, 1923; Korenchevsky and Carr, 1924]. These diets contained an abundant amount of phosphorus, *i.e.* 0·51 % in the fresh paste, and differed only in their calcium content. $-$ A diet contained an adequate amount of calcium (0·26 % in fresh paste), $-$ A_2 diet an excessive amount of calcium (0·52 % in fresh paste). These different diets were employed to ascertain if an excessive ingestion of calcium might not affect the results obtained.

In two experiments, Nos. 750 and 751 (Table I), the amount of calcium in the $-$ A diet was increased, not by the addition of $CaCO_3$ as was done in $-$ A_2 diet, but by the addition of calcium glycerophosphate, so that the fresh paste contained about 1·6 % of this salt. This diet contained 0·47 % Ca and 0·68 % P in the fresh paste.

The technique of the experiments was, on the whole, similar to that used in our previous investigations. The control rats always belonged to the same litter as those on glycerophosphate salts. We had great difficulty to choose a suitable preparation of calcium glycerophosphate. Grosser used Kahlbaum's salt which was soluble to about 5–6 %. But the salts now prepared by this firm as well as by English firms are only about 2·5 % soluble and are of different texture, and, when injected into rats, very often produce indurations followed by ulcerations. We were fortunate enough to obtain some of Kahlbaum's pre-war salt, which was 6 % soluble in water. This specimen of calcium glycerophosphate when injected in a 3 % watery solution was usually supported well by the rats.

As calcium glycerophosphate is more soluble in cold than in hot water it is difficult to sterilise a solution of it. Moreover we found that when dissolved in cold water, it becomes partly precipitated at 37·5° after about 24 hours from a 4 % solution and from a 5 % solution after about 4–5 hours. From this it is to be presumed that some precipitation probably occurs when such concentrations are injected subcutaneously. This probably explains the cause of the indurations and ulcerations produced, especially easily in young and weak rats on deficient diets, when 4 and 5 % solutions of this salt are injected subcutaneously. Rats on a normal diet or those put on deficient diets at an older age support injections of 4 % solutions of Kahlbaum's calcium glycerophosphate much better.

In our experiments a 3 % solution warmed to about 37° proved the most suitable. 1–2 cc. of this solution, according to the size of the animal, were

injected slowly into the subcutaneous tissue four to five times a week, the place of injection being altered each time. Without all these precautions indurations and necrosis were produced at the places of injection. In some of our first experiments on larger animals we began by injecting 3·5–4 % solutions which we finally replaced by 3 %. Kahlbaum's calcium glycerophosphate which we used contained 14·72 % Ca and 11·34 % P.

We also used Kahlbaum's sodium glycerophosphate which contained 10·006 % P. A solution was prepared for injection, 1 cc. of which contained the same amount of phosphorus as did 1 cc. of the calcium glycerophosphate solution used for corresponding animals of the same litter. For example, when animals from one group of the litter were injected with a 3 % solution of calcium glycerophosphate, others from the same litter were injected with a 3·4 % solution of the sodium salt (in the experiments Nos. 833, 845, 1163, 1007, 1041). As sodium glycerophosphate may possibly be excreted by the body more rapidly than the calcium salt, in experiment No. 1204 (Table II) the rats were injected twice daily with a 6 % solution of sodium glycerophosphate. In this way we tried to keep the concentration of the sodium salt in the body as high as possible. The strength of the sodium dihydrogen phosphate (NaH_2PO_4) solution was determined on the same principle as that of the sodium glycerophosphate solution. In experiments Nos. 1226 and 1233, 2·35 % solutions of the sodium phosphate and 6 % of sodium glycerophosphate were injected once daily. Neither of these sodium salts produced any harmful local or general effects upon the rats injected.

Details of the experiments and of the results obtained are clear from Tables I–III. The theoretical calcium requirement during the period of injection, viz. 1 % of the gain in weight during that time, based on a method mentioned elsewhere [Korenchevsky, 1922], was calculated in some experiments so as to show what proportion of the calcium required normally for complete calcification of the skeleton was provided in the form of calcium glycerophosphate.

III. Ingestion of Calcium Glycerophosphate.

The influence of the ingestion of calcium glycerophosphate was studied in two small litters each containing two control rats fed on – A diet and two fed on this same diet with the addition of calcium glycerophosphate (see Table I). No difference was observed between the chemical composition of the skeleton of the animals in these two groups; the ingestion of a nearly doubled amount of calcium in the form of calcium glycerophosphate did not influence calcification at all.

The calcium ingested by the rats on – A diet and more especially by those on the calcium glycerophosphate diet was well in excess of the theoretical requirement. The average total consumption of calcium by a rat on the calcium glycerophosphate diet from litter 750 was 2·5 g. and from litter 751 was 2·4 g.

The ratio of Ca : P (1 : 1·45) in the calcium glycerophosphate diet was a good one for normal calcium metabolism.

Table I. *The influence of the ingestion of calcium glycerophosphate upon the growth and skeleton of rats kept on – A diet.*

No. of experiment	No. of rats	Duration of administration of Ca G.P. in days	Total amount of Ca administered as Ca G.P. g.	Final average weight of rats in g.		In bones			Approximate theoretical calcium requirement in g.
				Males	Females	H_2O %	Fresh Ca %	Dry Ca %	
750 controls	2	—	—	102	94	49·9	8·4	16·8	—
,, Ca G.P.	2	46	1·175	116	72	49·6	8·4	16·6	0·48
751 controls	2	—	—	126	97	46·7	8·9	16·8	—
,, Ca G.P.	2	39	1·071	105	98	48·3	8·8	16·9	0·47
			Normal rats of the same age:						
	—	—	—	—	—	43·8	11·5	20·5	—

Note. In this and the other tables the following abbreviations are used:

Ca G.P. Calcium glycerophosphate.
Na G.P. Sodium glycerophosphate.
Na P. Sodium dihydrogen phosphate (NaH_2PO_4).

Therefore (1) this experiment confirms Korenchevsky's [1923] previous conclusions that the ingestion of increased quantities of calcium does not improve the rachitic calcium impoverishment of the skeleton of rats on a diet deficient in fat-soluble factor, provided that this diet already contains an adequate amount of calcium.

(2) This experiment also shows that calcium glycerophosphate is of no more value than other calcium salts for calcification of the skeleton when taken *per os*.

IV. Injection of Calcium Glycerophosphate into Rats kept on – A diet.

This series consisted of five experiments conducted on 37 rats, 22 of which were injected and 15 were controls (see Table II).

The experiment was started before the growth of the animals was arrested, because otherwise it was difficult to keep the animals alive for a sufficient length of time to give the necessary number of injections, although with rats on – A_2 diet this was done successfully in four cases (see below). The period of injection lasted from 1–3 months. The injections were begun when the animals commenced to grow more slowly and all gained in weight during the injection period (see Table). Therefore the results obtained might be attributed more to the preventive influence of the injections than to their power of curing skeletal disorders already developed. Theoretically one would expect that if the store of fat-soluble factor was not completely exhausted the injected calcium could be retained more easily by the organism and deposited in the skeleton. However, as will be shown later, there was not any special difference between the results obtained from rats which had ceased to grow and those which continued to grow during the period of injection.

When the total amount of calcium glycerophosphate injected was small, 0·08 g. and less, no influence was produced upon the skeletons of the rats (see litter 803, Table II). But when a larger amount (total of 0·17–0·22 g.) of this salt was injected in litters 787, 800, 809 and 811, calcification of the bones of these animals was much better than that of the control animals from the same litter. Comparing the injected with the control animals there was an average increase in the former of 13·4 % in calcium content of the fresh skeleton (minimal 5·4 % in litter 809 and maximal 20·7 % in litter 811) and of 14·6 % in the dry bones (minimal 8·3 % in litter 809 and maximal 20·9 % in litter 787). The water content of the bones changed very little, the average being an increase of 1·1 %.

Table II. *The influence of injections of calcium and sodium glycerophosphate and sodium dihydrogen phosphate upon the growth and skeleton of rats kept on* — A *diet* (*Ca content of fresh diet* 0·26 %).

No. of the experiment and of group	No. of rats	Gain (+) or loss (−) in weight in g. during period of injections		Total amount of Ca and P injected g.		In bones			Approximate theoretical calcium requirement in g.
		Male	Female	Ca	P	H_2O %	Fresh Ca %	Dry Ca %	
803 control ...	4	+29	+ 1	—	—	45·6	8·8	16·1	—
„ Ca G.P. ...	7	+29	+ 9	0·04–0·08	—	48·6	8·4	16·3	0·1–0·3
809 control ...	4	+33	+26	—	—	45·2	9·3	16·9	—
„ Ca G.P. ...	4	+33	+29	0·17	0·13	46·3	9·8	18·3	0·3
787 control ...	2	+23	+19	—	—	45·1	8·2	14·7	—
„ Ca G.P. ...	4	+35	+11	0·18	0·14	44·9	9·8	17·8	0·25
800 control ...	3	+50	+41	—	—	39·7	11·1	18·4	—
„ Ca G.P. ...	3	+71	+50	0·20	0·15	40·7	12·0	20·2	0·6
811 control ...	2	+19	+30	—	—	41·0	8·2	14·0	—
„ Ca G.P. ...	4	+27	+19	0·22	0·16	40·6	9·9	16·7	0·23
1204 control ...	4	+24	+28	—	—	45·1	8·5	15·4	—
„ 3·4 % Na G.P.	3	+49	+27	—	0·11	45·6	8·9	16·4	—
„ 6·0 % Na G.P.	4	+45	+23	—	0·29	46·2	8·7	16·1	—
1226 control ...	2	+27	+ 7	—	—	49·8	7·4	14·7	—
„ 2·35 % Na P.	3	+12	+17	—	0·13	47·1	7·7	14·4	—
„ 6 % Na G.P.	2	+10	+ 5	—	0·13	47·4	7·7	14·7	—
Normal calcification at the ages of litters:									
Nos. 803, 1204 and 1226	—	—	—	—	—	43·8	11·5	20·5	—
Nos. 787, 800, 809 and 811	—	—	—	—	—	36·8	14·2	22·4	—

The calcium content of the skeletons of the injected animals never reached the level found in rats of the same age on a normal diet (see Table II). This was especially clear in one litter (811) in which the amount of calcium injected was nearly as much as the total calcium requirement of the rats during this period. It is probable that some of the injected calcium glycerophosphate is excreted by the body before there has been time for the deposition of the calcium in the skeleton. *A priori*, more satisfactory results would be expected were it possible to inject larger amounts of calcium. Technically it was difficult to inject a larger amount of the salt (1) because the rats on the deficient diet become emaciated rather quickly and die and (2) because it is impossible to inject a saturated solution of the salt.

The daily injection of such large volumes of calcium glycerophosphate solution as 1·5 cc. and 2 cc. (for larger animals) did not impair the general nourishment of the rats. On the contrary, in several cases the weights of the treated animals were sometimes greater than those of the controls. From the weight figures it is difficult to draw any definite conclusion about the influence of these injections upon the growth.

V. Injection of Calcium Glycerophosphate into Rats kept on — A_2 diet.

Sixty-two rats belonging to nine litters were used for this series of experiments, 35 animals were injected and 27 remained as controls (see Table III). The duration of the injection period varied from about 1 to 1½ months, during which time 0·1–0·16 g. Ca and 0·08–0·12 g. P were injected in the form of calcium glycerophosphate solution into each animal. The results obtained were similar to and even more pronounced than those of the previous series in spite of the doubled amount of calcium in the diet. In four (869, 879, 833 and 845) of the nine litters growth practically ceased; sometimes it increased a little during the first half of the injection period and nearly always decreased during the second half. In the other five litters (1213, 1169, 1163, 1007 and 1041) the rats grew during most of the experiment, but towards the end the growth was slower and in several cases stopped. There was no essential difference between the results obtained in these two groups of litters. It is difficult to draw any conclusions concerning the effect of the injections upon the growth of the animals.

The least pronounced influence of the injections upon the skeleton was noticed in litter 1213, to each animal of which was only administered a small amount of calcium glycerophosphate containing 0·1 g. Ca and 0·08 g. P. In litter 1041 the deficient diet produced only a slight calcium impoverishment of the skeleton and the rats grew well owing to the good food given to the mother during pregnancy and lactation. One rat even became pregnant, which is unusual for animals on a diet deficient in fat-soluble factor. These facts suggest that there was a greater storage of fat-soluble factor in these animals than in those of other litters and so a fairly high calcification of the skeleton resulted in spite of the deficient diet. Because of this the injections caused the calcification of the skeleton of the treated animals to be nearly normal. The calcium content of the dry bone was normal, but the water content was greater, being 42·1 % instead of 39 %, making the calcium in the fresh bone lower than that of the normal animal (12·5 % instead of about 13·2 %).

The following figures show the average percentage changes produced by the injections in all the other litters. The calcium content of dry bone was 18·4 % (minimal 10·9 % in litter 1007 and maximal 27·7 % in litter 845) higher in the injected animals than in the controls. Similarly in fresh bone there was an increase of 16·5 % in the calcium content (minimal 7·2 % in litter 869 and maximal 27·5 % in litter 845). In most cases the water content

of the bones was influenced slightly, being higher by about 2 % than that of the controls. Only in one litter, 869, did the skeletons of the injected animals contain much more water (12·1 %) than the controls.

Table III. *The influence of injections of calcium and sodium glycerophosphates and sodium dihydrogen phosphate upon the growth and skeleton of rats kept on – A_2 diet (Ca content of fresh diet 0·52 %).*

No. of experiment and of group	No. of rats	Gain (+) or loss (–) in weight in grams during period of injections		Total amount of Ca and P injected g.		In bones		
		Male	Female	Ca	P	H_2O %	Fresh Ca %	Dry Ca %
1213 control ...	2	+22	+27	—	—	48·0	8·6	16·4
„ Ca G.P. ...	3	+26	+25	0·1	0·08	50·2	9·0	18·0
869 control ...	4	0	– 5	—	—	44·6	8·3	14·9
„ Ca G.P. ...	6	– 1	– 3	0·15	0·11	50·0	8·9	17·8
879 control ...	3	–13	– 6	—	—	44·2	8·3	14·9
„ Ca G.P. ...	4	—	+ 2	0·15	0·11	46·3	9·6	17·9
1169 control ...	3	+21	+22	—	—	45·6	9·2	16·9
„ Ca G.P. ...	4	+33	+29	0·15	0·11	43·7	11·1	19·4
833 control ...	3	– 5	– 9	—	—	47·2	8·0	15·1
„ Na G.P. ...	4	– 8	– 5	—	0·11	47·4	8·4	15·9
„ Ca G.P. ...	4	0	– 6	0·15	0·11	48·7	9·1	17·8
845 control ...	3	– 4	– 9	—	—	51·5	6·9	14·1
„ Na G.P. ...	3	– 1	– 4	—	0·11	49·2	8·0	15·8
„ Ca G.P. ...	3	– 6	+ 1	0·15	0·11	51·2	8·8	18·0
1163 control ...	4	—	+12	—	—	46·6	8·7	16·2
„ Na G.P. ...	4	—	+17	—	0·11	44·6	9·8	17·6
„ Ca G.P. ...	4	+31	—	0·15	0·11	47·1	10·1	19·1
1007 control ...	2	+30	+24	—	—	45·3	10·5	19·2
„ Na G.P. ...	3	+26	+13	—	0·12	42·2	11·4	19·7
„ Ca G.P. ...	3	+28	+12	0·16	0·12	43·9	12·0	21·3
1041 control ...	3	+68	+92*	—	—	43·2	11·5	20·2
„ Na G.P. ...	3	+69	+65	—	0·12	42·9	11·5	20·4
„ Ca G.P. ...	4	+90	+49	0·16	0·12	42·1	12·5	21·5
1233 control ...	2	—	+18	—	—	47·4	7·6	14·5
„ 2·35 % Na P.	2	—	+19	—	0·13	48·5	8·0	15·4
„ 6 % Na G.P.	2	+27	+20	—	0·13	51·8	7·3	15·2
Normal calcification at the ages of litters:								
Nos. 1213 and 869	—	—	—	—	—	43·8	11·5	20·5
Nos. 833, 845, 879, 1007, 1041, 1163 and 1169 ...	—	—	—	—	—	39·0	13·2	21·5

* This was one female rat which had become pregnant.

Table IV. *Percentage changes above (+) or below (–) the controls in the composition of the skeleton of rats injected with sodium and calcium glycerophosphates.*

No. of litter	Na G.P. group In bones			Ca G.P. group In bones		
	H_2O %	Fresh Ca %	Dry Ca %	H_2O %	Fresh Ca %	Dry Ca %
833	+0·4	+ 5·0	+ 5·3	+3·2	+13·7	+17·9
845	–4·5	+16·0	+12·1	–0·6	+27·5	+27·7
1163	–4·3	+12·0	+ 8·6	+1·1	+16·1	+17·9
1007	–6·8	+ 8·6	+ 2·6	–3·1	+14·3	+10·9
1041	–0·7	0	+ 1·0	–2·5	+ 8·7	+ 6·4
Average	–3·2	+ 8·4	+ 5·7	–0·4	+16·1	+16·2

It is interesting to note that in most cases the water content of the bones of the rats injected with calcium glycerophosphate is either the same or higher than that of the controls notwithstanding the improvement in the calcification. This shows that, although calcium glycerophosphate caused an increase in the calcium deposited in the skeleton of rats on a diet deficient in fat-soluble factor, it did not produce such a general improvement in the general composition of the bones as is caused, for instance, by the fat-soluble factor.

VI. Injection of Sodium Glycerophosphate and of Sodium Dihydrogen Phosphate into Rats kept on − A or − A_2 diets.

This series consisted of eight experiments in which 33 rats were injected. In five of these experiments, Nos. 833, 845, 1163, 1007 and 1041, the same 15 rats were used as controls as were mentioned above in Section V; each of these five litters was divided into three groups: controls, those injected with calcium, and those injected with sodium glycerophosphate (Table III). The sixth experiment, litter No. 1204, was divided into the following three groups: 4 control rats, 3 injected with 1·0 cc. of 3·4 % solution of sodium glycerophosphate once daily, and 4 injected with 1·0 cc. of 6 % solution of the same salt twice daily. This latter series of injections was made in order that the animals might retain as much sodium glycerophosphate as possible. The aim of the last two experiments, Nos. 1226 and 1233, was to compare the influence upon the calcification of the skeleton of injections of sodium glycerophosphate with those of sodium dihydrogen phosphate. Four rats were used as controls, 5 were injected with 1 cc. of 2·35 % solution of sodium dihydrogen phosphate and 4 rats with 1 cc. 6 % solution of sodium glycerophosphate once daily. Two (Nos 1204 and 1226) of these eight experiments were performed on rats kept on − A diet and the remaining six on rats kept on − A_2 diet. No difference was found in the results obtained from the injected rats on whichever of these two diets they were fed. If the influence of the injections of sodium glycerophosphate is examined in all the 28 rats treated, the following figures show the average percentage changes in the chemical composition of the bones above (+) or below (−) that of the controls.

The calcium content of dry bone was 5 % higher in the injected animals than in the controls (minimal 0 in litter 1226 and maximal + 12·1 % in litter 845). Similarly in fresh bone there was an average percentage increase of 5·5 % in the calcium content (minimal 0 in litter 1041 and maximal + 16 % in litter 845). The water content in all cases was slightly influenced, being on an average − 0·9 %. In five litters (Nos. 833, 1041, 1204, 1226 and 1233) the changes were within the usual fluctuations found in the calcification of the skeleton of rats kept on a diet deficient in fat-soluble factor.

Table IV is constructed to show the comparative value of calcium and sodium glycerophosphates in the calcification of the skeleton of rats kept on a deficient diet. In it are summarised the results obtained in those five litters

mentioned above which were divided into three groups: (1) control rats, (2) those injected with calcium glycerophosphate, and (3) those injected with sodium glycerophosphate. These groups were discussed separately above. When these figures are compared (Table IV) and most particularly the average figures, it will be seen quite clearly that the injection of the calcium salt always produced a more striking effect than that of the sodium salt. The effect on calcification of the former is approximately twice as great as that of the latter. In some litters this difference is found to be even greater (litters 833, 1041, and in the calcium content of the dry bone in litter 1007). When the average percentage changes in the composition of the skeleton of all 28 rats injected with sodium glycerophosphate are compared with those of 47 rats injected with about a corresponding amount of calcium glycerophosphate the difference becomes even more striking than that in Table IV. In the first group (sodium glycerophosphate) the average percentage changes in the bones when compared with the controls were as follows: H_2O − 0·9 %, Ca in fresh bone + 5·5 % and Ca in dry bone + 5·0 %, in the second group (calcium glycerophosphate) these changes were + 0·6 %, + 15·3 % and + 17·1 % respectively.

Unfortunately, up to the present time, our experiments with sodium dihydrogen phosphate are not conclusive owing to the few rats used for the injection treatment and to the small changes and contradictory results obtained. On the basis of our previous experiences such results may only be the fluctuations usually obtained in the composition of the skeleton of rats kept on this deficient diet. In experiment 1226 (Table II) sodium phosphate did not produce any influence upon the calcification of the skeleton, whereas in experiment 1233 (Table III) the animals injected showed a slightly higher calcium content of the skeleton than the controls. In neither litter was there any appreciable difference between the effect of sodium dihydrogen phosphate and that of sodium glycerophosphate.

In a paper published simultaneously with this, Robison and Soames have found evidence in one experiment of a slight increase in the calcification of the skeleton of rats kept on − A diet after subcutaneous injections of sodium glycerophosphate, while no such increase was found after the injection of sodium phosphate [1925].

We cannot enter into a theoretical explanation of the mechanism resulting in the increase of the calcification of the skeleton after subcutaneous injections of these glycerophosphates. Robison and his co-workers [1923, 1924], who have made many interesting experiments on this subject at the Lister Institute, put forward the following theory. There is present in ossifying cartilage an enzyme which hydrolyses certain phosphoric esters including glycerophosphoric ester. By this reaction the concentration of inorganic phosphate is increased locally and calcium phosphate deposits in the bone.

VII. Histological investigation.

In all control rats kept on – A or – A_2 diets we found slight rickets with osteoporosis or even only the latter, because the mother rats were well fed during pregnancy and lactation. As we have mentioned several times before, it is difficult to draw any definite conclusions concerning slight histological changes observed in experimental rats, when the controls show only mild rickets and osteoporosis. In rats on – A diet the histological pictures vary sometimes to a large degree principally because of more or less pronounced osteoporosis. Notwithstanding the great difference in the histological pictures, the chemical composition of the skeleton of such rats is very similar in most animals belonging to the same litter. Therefore, when only a slight influence of some agent is applied to rats on this deficient diet, the chemical results are found much more reliable and objective than the histological ones.

Bearing these limitations in mind, we may summarise the histological results of the present experiments shortly as follows. Injections of sodium phosphate and sodium glycerophosphate as well as the ingestion of calcium glycerophosphate did not alter the histological structure of the bone of the rats. When the total amount of calcium glycerophosphate injected was not smaller than 0·15 g., a definite improvement was observed in the pathological changes in the bones of about half of these animals.

Therefore, although in a lesser degree, the histological results corroborated those obtained chemically.

Conclusions.

1. The ingestion of calcium glycerophosphate, like that of other calcium salts, did not cure the rachitic calcium impoverishment of the skeleton found in rats kept on a diet deficient in fat-soluble factor only.

2. Subcutaneous injections of a watery solution of calcium glycerophosphate considerably increased the degree of calcification of the skeleton of rats kept on the same deficient diet.

3. The water content of the bones of the rats injected with calcium glycerophosphate was the same as, or higher than, that of the controls in spite of the improvement in the calcification.

4. Similar injections of sodium glycerophosphate produced in most cases the same effect as the calcium salt, but in a much less degree.

5. In about 50 % of the rats injected with calcium glycerophosphate a slight improvement of the rachitic changes in the skeleton was found histologically, which was not observed in animals injected with sodium glycerophosphate.

6. Therefore, the presence of calcium in the compound calcium glycerophosphate injected into rats on a diet deficient in fat-soluble factor seems to be of considerable importance for the greater deposition of calcium in the skeleton.

7. Calcium glycerophosphate may probably be considered as a weak therapeutical agent in the treatment of rickets produced by a deficiency of fat-soluble factor, as compared with cod-liver oil, after the ingestion of which the composition and structure of the rachitic bone of such animals returns to the normal.

8. These facts suggest a difference in the mechanisms of calcification induced in the skeleton of rats on a diet deficient in fat-soluble factor by administration of calcium glycerophosphate and of cod-liver oil respectively.

9. Our results agree with those of Grosser, who showed some retention of calcium in rachitic children after subcutaneous injections of calcium glycerophosphate.

10. No change or only a small increase in the calcification of the skeleton compared with that of controls was found in 5 rats injected with sodium dihydrogen phosphate. This increase was within the limits of the usual fluctuations observed in rats kept on the deficient diet.

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XVI. FURTHER EXPERIMENTS ON THE INFLUENCE OF THE PARENTS' DIET UPON THE YOUNG.

III. THE INFLUENCE UPON THE YOUNG OF AN EXCESSIVE AMOUNT OF CALCIUM IN THE MOTHER'S DIET DURING PREGNANCY.

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This is an addition to our previous communication [Korenchevsky and Carr, 1924] in which we omitted to take into consideration the results of some experiments we had published elsewhere [1923, 1]. In these experiments the mothers of 18 rats, belonging to three litters, Nos. 621, 624 and 646, were fed during pregnancy on N_1 (1 mother) or D_2 (2 mothers) diets (see Table, p. 113). Both these diets contained cod-liver oil. The calcium content of the former diet was adequate, but not excessive (0·26 % in fresh diet). In the latter most of the ingredients were given separately, and some in the form of paste (in particular cod-liver oil and calcium salts). Therefore, although this diet included calcium salts as well, the latter were ingested unevenly owing to the possibility of the rats selecting components of the diet poor in calcium. Roughly estimated, the rats consumed in this diet about the same amount of calcium as they did in N_1 diet, containing 0·26 % Ca.

In this paper the results obtained with these 3 litters are compared with those described in the previous paper, obtained with litters, whose mothers were fed during pregnancy on different diets. The diet of the mothers, though differing before and during pregnancy, was the same during lactation. The mothers during lactation, and the young rats after weaning, were fed on a diet deficient in fat-soluble factor (– A). The chief characteristics of the other diets mentioned in this communication are as follows. Diet N_2 was similar to the diet N_1, but differed from the latter by containing twice the amount of calcium. Diet NB_3 contained three times the amount of calcium present in NB. In both these diets the only source of fat-soluble factor was butter. All the diets used except D_2 (see above) were given in the form of a paste, by which means the amount of each ingredient eaten could be measured accurately. The technique of the experiments and details of the diets were described in our previous communications [1923, 1; 1924].

The results obtained are set forth in the Table in average figures. They have led us to *modify to some extent the first and third conclusions in our previous paper* [1924].

There was no difference between the weight or the chemical composition of the skeleton of the NB and NB_3 groups of rats (see Table). Therefore, the addition of an excess of calcium to the normal diet (containing butter as the only source of fat-soluble factor), given to the mothers during pregnancy, did not improve the condition of the young nor diminish the severity of rachitic changes in their skeletons[1].

Table.

The influence of different diets of mothers during pregnancy upon the young. The mother's diet during lactation as well as that of the young after weaning was deficient in fat-soluble factor.

Mother's diet during pregnancy	Amount of Ca % in fresh paste	Presence (+) or absence (−) of fat-soluble factor in the diet	No. of rats used for the average	Final weight of young in g. ♂	Final weight of young in g. ♀	In bones H_2O %	In bones Fresh Ca %	In bones Dry Ca %
At the day of weaning (24 days old).								
N_2	0·52	+(cod-liver oil and butter)	10	38	34	58·4	6·2	15·0
NB_3	0·77	+(butter only)	4	34	34	62·2	4·8	12·7
NB	0·26	+(butter only)	63	31	30	61·4	5·0	12·7
−A	0·26	−(cottonseed oil)	5	19	19	68·3	2·6	8·2
When 65 days old.								
N_2	0·52	+(cod-liver oil and butter)	25	90	85	44·5	9·3	16·6
N_1 (or D_2)	0·26	+(cod-liver oil and butter)	18	55	44	48·4	6·4	12·4
NB_3	0·77	+(butter only)	6	53	62	52·1	6·5	13·7
NB	0·26	+(butter only)	62	62	53	50·9	6·8	13·8
Normal rats about 65 days old			19	140	108	43·8	11·5	20·5

However, when the mother's diet during pregnancy contained an excess of calcium *and cod-liver oil*, then the young rats showed less disorders of general nutrition and rachitic changes in the skeleton. The average figures of the weight and calcification of the skeleton of the N_1 group of rats, belonging to the litters 621, 624 and 646, and those of the N_2 group showed this quite definitely (see Table). These results emphasise the importance for the young not only of an excess of fat-soluble factor in the form of cod-liver oil, but also of ample calcium in the mother's diet during pregnancy.

It was also recorded that *at the day of birth* the Ca and P content in the young was nearly the same, irrespective of the mother's previous diet [Korenchevsky and Carr, 1923, 2]. Therefore our observations support the hypothesis, already suggested by us [1923, 1]. Our present view with regard to the influence of calcium in the mother's diet during pregnancy can be summarised as follows.

Calcium is of importance not only to the structure of the skeleton: it is a necessary component of all cells, and the nuclei contain an especially large

[1] However, when interpreting these results, it is necessary to bear in mind that butter can vary much in its fat-soluble factor content.

amount of it. On the calcium content in the tissues and environment depend the working capacity and the different excitability and permeability of blood vessels, the coagulation of the blood, the resistance to certain toxins, etc. It may, therefore, be assumed that the intake of large quantities of calcium may improve the composition and functions of the mother's organism during the complex process of pregnancy, when the production of new tissues is markedly increased and the whole metabolism is altered. It is natural to expect that a more perfect organism will produce more healthy offspring, more capable of resisting deleterious pathological factors, in particular a rickets-producing — A diet.

Probably the presence of cod-liver oil in the mother's diet during pregnancy helps to make use of the excess of calcium and favourably influences the nutrition and calcium metabolism of the young.

There are indications that feeding animals with cod-liver oil is one of the most efficient ways to increase the store of fat-soluble factor in the organism. Coward, Lush and Palmer [1923] and Goldblatt and Soames [1923] stated that the storage of growth-promoting and anti-rachitic factors in the liver of rats varies very much with the concentration of these factors in the diet. The latter authors found an increase of growth-promoting factor in the livers of those animals which were fed previously with a cod-liver oil diet as compared with those of rats fed on a butter diet.

These experiments of ours add further to the explanation, already given [Korenchevsky and Carr, 1924] with regard to the divergence of results obtained by us and by Hess and Weinstock [1924, 1]. These latter workers in their interesting investigation of the anti-rachitic effect of addition of cod-liver oil to the diet of pregnant rats used a mixed diet, which they defined "ample, although not an optimal dietary" (p. 1). Its calcium content was not estimated but seemed to be rather low. Possibly, owing to this also the results obtained by Hess and Weinstock were similar to those of ours obtained in the N_1 and D_2 groups of rats.

Here also we refer to Korenchevsky's [1922] experiments: the most severe changes of the skeleton, practically identical with rickets, were produced in young rats, which, as well as their mothers *during lactation*, were fed on a diet deficient in calcium, but containing an *ample amount of cod-liver oil.*

Hess and Weinstock [1924, 2] do not give any data with regard to the diet of the pregnant women to whom cod-liver oil was given during pregnancy with the aim of protecting their children from rickets. In their recent experiments on rats [1924, 2] cod-liver oil was not added to the diet of the pregnant animals, nor was the amount of consumed calcium determined. In their experiments the diet of the rats during pregnancy consisted of different ingredients, given separately, not in the form of a paste, which made it difficult to determine what was consumed. However, owing to the differences in experimental technique, Hess and Weinstock themselves [1924, 1, p. 6] state that "it is difficult to compare the two series of experiments" (theirs and ours). Another

statement of these authors runs as follows [1924, 2, p. 1562]: "It may be stated as a principle that the resistance to rickets can be lessened by means of inadequacy of a mother's diet far more readily than a refractory condition can be brought about." Besides the results of our experiments on the influence of maternal diet upon the young, experiments of Kennedy and Dutcher [1922], Drummond, Coward and Watson [1921], Reyher [1924] and Luce [1924] showed quite definitely the great variations in growth-promoting or anti-rachitic properties of cow's milk and milk-fat, depending on the diet of the cow. Even without considering our experiments, it seems difficult to suppose that a varying amount of anti-rachitic factor in the maternal milk does not influence the resistance of the offspring to a rickets-producing diet deficient in the above factor.

Moore [1924] after giving cod-liver oil to two pregnant women observed much superior skeletal conditions in the babies born as compared with the rachitic disorders found in their previous children.

We feel that more numerous experiments are needed concerning the protective influence that the mother's feeding during pregnancy and lactation has upon the young. But we wish to emphasise once more that for these experiments and observations it is most essential to use *an optimal diet complete in every respect and to check the amount eaten*. As far as we can say at present, the presence of cod-liver oil and of an ample amount of calcium (in correct ratio with P) in such a diet is of great value.

Conclusions.

1. When the mother's "normal" diet during pregnancy was enriched with cod-liver oil and an excess of calcium, there was a marked decrease in the disorders of general nutrition and in the rachitic changes in the skeleton, produced in the young by a diet deficient in fat-soluble factor, even when the mother was also kept on the same insufficient diet during lactation.

2. This effect was not produced when the mother's "normal" diet during pregnancy contained butter as the only source of fat-soluble factor and was enriched by an excess of calcium.

3. Our experiments indicate a difference between the capability of cod-liver oil and butter to enable the animals to utilise an excess of calcium, present in the diet of the mother rats during pregnancy. At present it is impossible to state whether this difference is of a quantitative or qualitative nature owing to the large variations of anti-rachitic factor present in milk-fat.

4. Provided that the mother-rats' diet during pregnancy is complete in every respect and that the fat-soluble factor is present in the form of cod-liver oil, an ample amount of calcium in the maternal diet is of great importance for the young, kept after weaning on the above deficient diet.

A grant from the Medical Research Council and the hospitality of the Lister Institute have enabled us to carry out this work and to them our thanks are due. We wish also to express our sincere gratitude to Prof. C. J. Martin for his criticism of this work.

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XVII. THE PHOSPHORIC-ESTERASE OF BLOOD AT VARIOUS HYDROGEN ION CONCENTRATIONS.

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In a recent paper [Martland, Hansman and Robison, 1924] it was shown that when defibrinated blood is kept for 2–3 hours at 38° in the presence of small quantities of added acid, or in an atmosphere containing excess of CO_2, the concentration of inorganic phosphate is increased owing to enzymic hydrolysis of the phosphoric esters present in the blood. Conversely, additions of small amounts of alkali, or ventilation of the blood with CO_2-free air, gave rise under similar conditions to a synthesis of phosphoric ester with reduction in the amount of free phosphate, an observation which has been made independently by Lawaczeck [1924].

From these experiments it was suspected that the regulating mechanism which determines the activity of the esterase might prove to be the hydrogen ion concentration of the blood, but accurate estimations of p_H were not at that time made. The object of the present work was to make a closer study of this relationship, and to determine whether such changes in reaction as might be found to affect the equilibrium between the hydrolytic and synthetic activities of the enzyme are those which might occur in the blood of the living organism.

Experimental.

A method was devised by which blood at 38° might be kept at a stable p_H for a length of time sufficient for the study of the action of the phosphoric esterase. To the defibrinated whole blood of a rabbit $N/2$ sulphuric acid was added, drop by drop, in a measured amount sufficient to neutralise the plasma bicarbonate and yet insufficient for the production of acid haematin. The blood and acid were mixed and allowed to stand at room temperature for 5 minutes, to allow the hydrogen ion concentration within the corpuscles to come into equilibrium with that of the plasma, at the end of which time samples were withdrawn for estimation of p_H and of inorganic phosphate. The blood, contained in a large flask, was then kept at 38° for half an hour, during the whole of which time the flask was rotated rapidly so as to spread the blood in a thin film over the wall of the vessel, the air of the room being drawn over the blood at 5 minute intervals. By this means the CO_2 tension

of the blood was brought into equilibrium with that of the surrounding atmosphere.

Samples having been withdrawn at this point for estimation of p_H and free phosphate, the blood was divided into aliquot parts and different amounts of $N/2$ caustic soda added to each. The suitable quantities of alkali had been found empirically after a considerable number of experiments, the object being to bring the p_H of the blood to values lying closely on either side of the point at which synthesis and hydrolysis are in equilibrium. The p_H and inorganic phosphate were estimated 5 minutes after adding the alkali and again after standing, without shaking, at 38° for periods varying from half-an-hour to one-and-a-half hours. No experiment was prolonged beyond two hours from the killing of the animal, it having been found that the synthetic activity of the enzyme became markedly less after that time.

Estimations of blood sugar were made parallel with the p_H and phosphate determinations, in view of the possibility that the phosphoric ester may be present in the blood in combination with a reducing group. The p_H was estimated by the method of Dale and Evans [1920], the inorganic phosphate by that of Briggs [1922], and the sugar by the method of Hagedorn and Jensen [1923].

The results of three experiments are here set forth, the phosphate and sugar being expressed as mg. per 100 cc. of defibrinated whole blood (D).

Vol. of $N/2$ H_2SO_4 per 10 cc. D	Vol. of $N/2$ NaOH added after 30 minutes cc.	Time (mins. from beginning of experiment)	p_H	Sugar mg.	Inorganic phosphate mg. P	Hydrolysis (H) or Synthesis (S) of phosphoric ester mg. P
Exp. 1.						
0·7 cc.	—	5	<6·50	125	5·15	1·48 H.
		35	6·83	128	6·63	
0·7	0·3	40	7·13	127	6·62	1·89 H.
		95	7·16	134	8·51	
0·7	0·4	40	7·36	122	6·58	1·25 S.
		95	7·38	108	5·33	
0·7	0·5	40	7·48	123	6·62	1·40 S.
		95	7·51	92	5·22	
Exp. 2.						
0·7 cc.	—	5	<6·50	128	4·92	0·79 H.
		30	6·81	128	5·71	
0·7	0·3	35	7·15	126	5·53	0·72 H.
		80	7·19	130	6·25	
0·7	0·4	35	7·35	117	5·35	0·79 S.
		80	7·39	112	4·56	
0·7	0·5	35	7·50	129	5·33	0·84 S.
		80	7·55	112	4·49	
Exp. 3.						
0·7 cc.	—	5	<6·50	137	4·88	1·20 H.
		30	6·79	141	6·08	
0·7	0·3	35	7·0	134	6·07	0·83 H.
		60	7·09	140	6·27	
		120	7·11	135	6·90	
0·7	0·4	65	7·28	136	6·65	0·31 H.
		120	7·29	133	6·96	
0·7	0·5	65	7·39	141	6·06	0·76 S.
		120	7·41	120	5·30	

These tables show a definite relationship between p_H and inorganic phosphate. The variations in the blood sugar shed no new light on previous observations. At a p_H higher than 7·3 synthesis of organic phosphate and glycolysis proceed simultaneously, but at a p_H lower than 7·3 where hydrolysis of organic phosphate takes place, the blood sugar either remains constant or shows an increase so small as to be of doubtful significance. There is no evidence of any simple and direct connection between the esterification of phosphoric acid and the synthesis of non-reducing compounds from sugar. The occurrence of glycolysis in alkalosis has been noted by various workers, and has been correlated with an increase in the lactic acid of the blood [Anrep and Cannan, 1924].

Discussion of Results.

It will be seen that after the preliminary removal of CO_2 in the first half-hour, the p_H of a given blood sample shows only very slight change from the time at which the alkali was added to the end of the experiment, and it is legitimate to assume that the reaction has remained approximately constant throughout such a period. In Exp. 3, hydrolysis of phosphoric ester has taken place at a p_H of 7·09–7·11, as also at 7·28–7·29, while at 7·39–7·41 there is decrease of inorganic phosphate indicating synthesis of the ester. The other experiments give comparable results, hydrolysis of phosphoric ester being found at every p_H lower than 7·29, and synthesis above 7·35. The critical point at which hydrolytic and synthetic processes are in equilibrium appears to lie between 7·3 and 7·35. This corresponds closely to the mean average p_H found in normal blood by various workers. Cullen and Robinson [1923] give a mean of 7·345 in normal human subjects, while in normal rabbits the mean average p_H has been found to be 7·42 for arterial blood, 7·345 for venous blood [Hawkins, 1924].

A survey of recent literature brings to light a number of instances in which alterations in the p_H of the blood *in vivo* are associated with changes in the amount of inorganic phosphate similar to those found *in vitro* in these experiments. In the observations made on a human subject by Haldane, Wigglesworth and Woodrow [1924], the acidosis induced by breathing an atmosphere containing 6–7 % CO_2 was computed to correspond to a fall of 0·09 in arterial p_H, the inorganic phosphate of the blood showing an increase from 3·39 mg. to 4·82 mg. after one-and-a-half hours under these conditions. Conversely, in alkalosis induced by forced breathing maintained for long periods, causing a reduction of alveolar CO_2 to 12 mm. and a calculated rise of about 0·3 in arterial p_H, the free phosphate of the blood was diminished to 1·25 mg. or less per 100 cc.

Kay [1924], approaching the same subject from a different point of view, has shown that the acidosis produced by ingestion of ammonium chloride gives rise to an increase of inorganic phosphate accompanied by a decrease in the amount of that fraction of the phosphoric esters upon which the esterase

of the blood has been shown to act [Martland, Hansman and Robison, 1924].

The blood changes in ether anaesthesia are significant also of a close connection between increased hydrogen ion concentration and hydrolysis of organic phosphate. Van Slyke, Austin and Cullen [1922] have shown that a true acidosis occurs in this condition, and after 20 minutes' administration of ether a p_H as low as 7·07 has been recorded [Austin, Cullen, Gram and Robinson, 1924]. It has been noted that during a similar period of ether anaesthesia in rabbits the amount of inorganic phosphate is very strikingly increased [Martland and Robison, 1924].

In the terminal stages of nephritis accompanied by the so-called uraemic syndrome the association of acidosis with increase of inorganic phosphate in the blood is well established. A case is reported by Van Slyke [1921] in which Cullen, using an electrometric method, found a blood p_H as low as 6·95 in a man dying of advanced nephritis. The very marked rise in the free phosphate of the blood in such conditions has been observed by de Wesselow [1923], who attaches a grave clinical significance to this change in the phosphorus metabolism.

A relationship between acidosis and hydrolysis of organic phosphate is suggested by the work of Allan, Dickson and Markowitz [1924], who obtain a large increase of phosphates in the urine after subcutaneous injection of phloridzin in dogs. No estimations of the blood phosphates have been made in these experiments, but the amount of phosphorus eliminated is of such an order that it would be difficult to account for it without assuming an increase of inorganic phosphate in the blood. The blood p_H in phloridzinised dogs has been found by another observer to lie between 7·01 and 7·29 [Ets, 1924].

Recent work of Cavins [1924] gives rise to speculations as to whether the ketosis which occurs in carbohydrate starvation may not be concerned in a hydrolysis of phosphoric ester. In experiments on rachitic rats, Cavins has first depleted the serum of inorganic phosphate by feeding with a diet deficient in phosphate and in fat-soluble A. He has then, on fasting these animals, found an increase of free phosphate to an amount far in excess of the normal, this rise being accompanied by a cure of the rachitic condition. Control experiments on non-rachitic rats showed however no such marked hydrolysis of phosphoric ester on fasting, and it may be that factors other than the p_H of the blood have been brought into action. It would be of interest to discover whether a true "uncompensated" acidosis may not have been induced by the starvation of the rachitic animals, the alkali reserve of the normal rats having possibly been adequate to keep the blood p_H constant, or their store of glycogen having been sufficient to avert any extreme ketosis within the time-limit of the experiments.

In each of the conditions enumerated, acidosis goes hand in hand with an increase, alkalosis with a diminution, of free phosphate. It is impossible to avoid making the inference that these changes are of the same nature as those shown to occur *in vitro*, a supposition which emphasises the importance of further study of the phosphoric ester—esterase reaction of the blood.

SUMMARY.

In blood as a result of the activity of the phosphoric-esterase hydrolysis of the phosphoric ester was observed when the p_H fell below 7·3, synthesis at a p_H above 7·35.

This relationship is suggested as an explanation of certain changes in the phosphate content of the blood associated with acidosis and alkalosis, which occur in diverse conditions in the living organism.

I wish to express my thanks to Dr R. Robison for much helpful criticism and advice, and to Dr E. H. Lepper, whose experience in the detail of technique has been of invaluable aid in the determinations of blood p_H.

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XVIII. THE EARLY ACTION OF INSULIN IN THE DIABETIC.

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Even in the case of the normal animal opinion is divided as to the effect of insulin on the metabolism of carbohydrate. Macleod [1922] and others believe that the first action of insulin is to cause the storage of sugar as glycogen. Dudley and Marrian [1923], as the result of experiments on normal small animals, came to the conclusion that insulin does not cause glycogen to be stored.

The object of the present investigation was to attempt to throw some light on this subject as affecting the diabetic. It was planned to study the respiratory exchange and blood sugar levels in the diabetic as influenced by the administration of food with and without protection from insulin.

For this purpose we investigated the respiratory exchange of four severe cases of diabetes, three of whom were adult males and one a youth of 19 years of age.

That they were cases of a severe type is shown by blood sugar curves obtained from each of them after the administration of 25 grams of glucose. As judged by the trend of the curves, Case IV was the most severe, Cases I and II reacted similarly, Case III was the least severe of the series. The results obtained by the administration of glucose were on the whole confirmed by clinical examinations of the patients.

The general plan of the experiments was to administer a certain meal after a basal metabolism determination and blood sugar estimation had been made. The metabolism and blood sugar levels were followed at intervals of 15 to 30 minutes. The effect of insulin was determined by repeating the control experiment, except that 30 minutes before a given meal a dose of insulin was injected. We thus have comparative experiments which show the effect of insulin on meals of various components in cases of diabetes of varying severity. All experiments were performed in the morning, the patients having abstained from food for 12–14 hours previously. The basal metabolism determinations were made with the open circuit apparatus of C. V. Bailey [1921]. In order that the samples of air could be examined at leisure, they were collected in gas pipettes over mercury, as was done by Wolf and Hele [1914], and analysed at the close of the experiment. Over 100 basal metabolism estimations were performed on the four patients. The results are, on the whole, very uniform

[1] Assisted by a grant from the Medical Research Council.

and allow us to draw some conclusions regarding the early influence of insulin on the diabetic.

Case I. The urine on admission to the hospital contained 7·6% of glucose and gave strong qualitative tests for acetone and acetoacetic acid. The experiments were commenced after a period of nine days, in which time the patient was given sugar-free and fasting regimens. With a daily diet of five eggs, ten ounces of green vegetables, and $1\frac{3}{4}$ ounces of butter, the urine contained traces of sugar, not estimable quantitatively. Acetone was present. The first experiment, after a 14 hour fast, was done with a meal containing 12·04 grams of protein, 20·0 grams of fat, with a total heat value of 238·3 Calories. The ketogenic-anti-ketogenic ratio, applying Woodyatt's formula [1922], was 2·5 : 1·0. This high ratio asserted itself in the increase in acetone compounds after the meal. From Exp. 1 it will be seen that the basal metabolism was 13 per cent. below the Dubois [1916] normal. The effect of the protein and fat was to raise the metabolism 17 per cent. within three-quarters of an hour after the meal; in two hours and a quarter the metabolism began to fall. The CO_2 was also increased, but not proportionately to the O_2 absorbed. The lung ventilation followed somewhat closely the execretion of CO_2.

The respiratory quotient in the basal state was 0·754 but with the administration of food the quotient fell, to resume its higher value at the end of the morning. The consumption of 12 g. of protein and 20 g. of fat did not materially alter the quotient.

The effect of insulin and a similar meal given to this patient is seen in Exp. 2. The experiment was performed six days after the preceding. The higher sugar concentration in the urine is explained by the fact that the patient surreptitiously obtained some bread the day before, and this was not discovered until the tests had been commenced.

The basal metabolism starts practically from the same level as in the previous experiment, namely 12 %. There did not appear to be the same influence on the metabolism due to the carbohydrate consumed the day before, as the respiratory quotient was 0·705. Lusk [1922] has recently shown that the effect of a carbohydrate meal may be traced for over 20 hours, and our findings appear to corroborate those of the American worker.

There is a small but unmistakable rise in the oxidation of carbon-rich substances, probably carbohydrate, following the injection of insulin, but the blood sugar remains constant indicating that the carbohydrate consumed was drawn from other sources than the sugar of the blood, or that a constant replacement of sugar was being effected, resulting in the glucose level in the blood being maintained. The dynamic rise in metabolism as a result of food protected by insulin is not as great as without the hormone, nor does the lung ventilation follow so closely the CO_2 elimination. There is again a definite drop in the respiratory quotient following the ingestion of food, but what is of greater interest is that metabolism is transferred almost entirely to the combustion of protein and fat, since in the triangular diagrams of

Case I. Computed oxygen absorption in the basal state: *217 cc. per min.*

	Time	Per min. O_2 c.c.	Per min. B.M.R. %	Per min. CO_2 cc.	R.Q.	Litres Lung vent.	Blood % sugar	Urine % sugar	Urine % acetone	Remarks
Exp. 1	9.30	—	—	—	—	—	0·223	Trace	+	Absolute rest
	10.15	187	−13	141	0·754	5·19	—	—	—	
	10.30	—	—	—	—	—	—	—	—	P. 12 g., F. 20 g., C. 0 ingested
	11.0	—	—	—	—	—	0·206	—	—	Patient quiet
	11.20	226	+ 4	166	0·735	5·79	—	—	—	
	12.40	215	− 1	161	0·750	5·75	—	—	—	
	12.45	—	—	—	—	—	0·203	Trace	+ + +	
Exp. 2	9.10	—	—	—	—	—	0·262	0·2	3·7	Absolute rest
	9.40	191	−11	135	0·705	5·02	— (g. per 1000 cc.)			
	10.0	—	—	—	—	—	—	—	—	*Insulin* U. 10
	10·25	199	− 8	149	0·749	5·40				
	10.30	—	—	—	—	—	0·260			
	10.40	—	—	—	—	—	—	—	—	P. 12 g., F. 20 g., C. 0 ingested
	11·10	217	± 0	155	0·715	5·23	—	—	—	Patient quiet
	11.30	—	—	—	—	—	0·208	—	—	
	11·45	222	+ 2	166	0·740	5·43	—	—	—	
	12.15	219	+ 1	166	0·760	5·42	—	—	—	
	12.35	215	− 1	165	0·765	5·35	—	—	—	
	12·45	—	—	—	—	—	0·162	0·2	1·9	
Exp. 3	9.10	—	—	—	—	—	0·195	0	6·8	Absolute rest
	9.45	181	−16	129	0·710	5·05	—	—	—	
	10.0	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 g., ingested
	10.45	223	+ 3	157	0·705	5·63	—	—	—	
	11.0	—	—	—	—	—	0·187	—	—	Patient quiet
	11.15	233	+ 7	171	0·735	6·20	—	—	—	
	11.40	241	+11	182	0·755	6·37	—	—	—	
	12.20	232	+ 7	183	0·790	6·38	—	—	—	Becoming a little restless
	12.30	—	—	—	—	—	0·270	1·58	7·5	
Exp. 4	9.10	—	—	—	—	—	0·165	0·37	1·06	Absolute rest
	9.45	171	−21	121	0·710	5·09	—	—	—	
	10.0	—	—	—	—	—	—	—	—	*Insulin* U. 10
	10.25	189	−13	139	0·735	5·20	—			
	10.30	—	—	—	—	—	0·285	—	—	P. 16·4 g., F. 24·7 g., C. 27 g., ingested
	11.15	225	+ 3	160	0·710	5·54				
	11.30	—	—	—	—	—	0·221			
	11.45	220	+ 1	173	0·785	5·31				
	12.10	219	+ 1	172	0·783	5·37				
	12.30	212	− 2	160	0·755	5·32				
	12.45	—	—	—	—	—	0·247	0·43	0·87	
Exp. 5	9.10	—	—	—	—	—	0·115	0	0·096	Absolute rest
	9.40	173	−20	125	0·721	4·93	— (g. per 1000 cc.)			
	9.50	—	—	—	—	—	—	—	—	*Insulin* U. 15
	10.15	189	−13	143	0·755	5·18				
	10.20	—	—	—	—	—	0·100			
	10.30	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 g., ingested
	11.10	214	− 1	144	0·673	5·39	—	—	—	
	11.25	—	—	—	—	—	0·141	—	—	Patient quiet
	11.40	232	+ 7	165	0·711	5·61	—	—	—	
	12.5	231	+ 7	167	0·723	5·67	—	—	—	
	12.35	226	+ 4	165	0·732	5·64	—	—	—	
	12.40	—	—	—	—	—	0·146	0	0·119	

Case I (*continued*)

	Time	Per min. O_2 cc.	Per min. B.M.R. %	Per min. CO_2 cc.	R.Q.	Litres Lung vent.	Blood % sugar	Urine % sugar	Urine % acetone	Remarks
Exp. 6	9.10	—	—	—	—	—	0·187	0	0	Absolute rest
	9.45	198	− 8	144	0·730	5·23	—	—	—	*Insulin* U. 20
	9.55	—	—	—	—	—	—	—	—	
	10.20	190	−12	147	0·776	5·57				
	10.25	—	—	—	—	—	0·171			
	10.30	—	—	—	—	—	—	—	—	P. 6·6 g., F. 6·6 g., C. 40·5 g., ingested
	11.0	218	+ 1	159	0·730	5·65	—	—	—	Patient quiet
	11.5	—	—	—	—	—	0·265	—	—	
	11.30	212	− 2	167	0·788	5·60	—	—	—	
	12.0	213	− 2	164	0·772	5·55	—	—	—	
	12.40	201	− 7	161	0·800	5·63	—	—	—	
	12.45	—	—	—	—	—	0·216	0	0	
Exp. 7	9.10	—	—	—	—	—	0·156	0	Trace	*Computed* B.M.R. 223 cc. O_2
	10.10	229	+ 3	165	0·723	6·57				
	10.15	—	—	—	—	—	—	—	—	P. 6·6 g., F. 6·6 g., C. 40 g., ingested
	10.55	—	—	—	—	—	0·174	—	—	Patient quiet
	11.0	220	− 2	150	0·685	5·52	—	—	—	
	11.30	247	+10	188	0·763	6·41	—	—	—	
	12.0	244	+ 9	206	0·846	6·39	—	—	—	
	12.30	239	+ 7	186	0·780	6·52	—	—	—	
	12.45	—	—	—	—	—	0·266	1·2	Trace	

Case II. Computed oxygen absorption in the basal state: *204 cc. per min.*

	Time	Per min. O_2 cc.	Per min. B.M.R. %	Per min. CO_2 cc.	R.Q.	Litres lung vent.	Blood % sugar	Urine % sugar	Urine % acetone	Remarks
Exp. 10	9.20	—	—	—	—	—	0·090	0	Trace	Absolute rest
	10.5	186	− 9	135	0·726	5·74	—	—	—	
	10.20	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 g., ingested
	11.0	210	+ 3	147	0·701	6·02	—	—	—	Patient quiet
	—	—	—	—	—	—	0·130	—	—	
	11.40	214	+ 5	159	0·743	6·77	—	—	—	
	12.15	213	+ 4	165	0·777	6·70	—	—	—	
	12.50	218	+ 7	165	0·756	6·63	—	—	—	
	1.0	—	—	—	—	—	0·232	Trace	Trace	
Exp. 11	9.10	—	—	—	—	—	0·114	0	0	Absolute rest
	9.45	187	− 8	134	0·715	5·51	—	—	—	*Insulin* U. 15
	9.50	—	—	—	—	—	—	—	—	
	10.15	174	−14	128	0·732	5·27				
	10.25	—	—	—	—	—	0·106			
	10.35	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 g., ingested
	11.15	201	− 1	144	0·714	5·48	—	—	—	Patient quiet
	11.20	—	—	—	—	—	0·115	—	—	
	11.40	199	− 2	147	0·738	5·53	—	—	—	
	12.10	202	− 1	156	0·775	5·59	—	—	—	
	12.40	202	− 1	157	0·779	6·01	—	—	—	
	12.45	—	—	—	—	—	0·116	0	0	

Dubois the oxidation of carbohydrate takes very little part in a metabolism the respiratory quotient of which is 0·715. It will be noticed, however, that the respiratory quotient rose steadily, the final figure being above that found in the companion experiment without insulin.

Disregarding any calculations as to the relationships between the blood sugar levels and the respiratory quotient, one may only say that the acetone concentration in the urine was less at the end of the experiment, indicating a more efficient combustion of fat, possibly due to the utilisation of carbohydrate as the result of the use of insulin.

Comparing the results obtained in the two experiments, it will be seen that the immediate effect of insulin is to increase the combustion of carbohydrate in the post absorptive state. Following the ingestion of the diet, the combustion is apparently transferred to the combustion of protein and fat, since the respiratory quotient falls at first, reaching a higher level at the end of 2½ hours when assisted by insulin than without it. What also is noteworthy is that the total metabolism following the ingestion of food and insulin is lower than without the hormone.

The next three experiments are concerned with the metabolism of the diabetic consuming meals containing protein, fat and carbohydrate. The effect of varying the amounts of insulin injected was investigated. The protocols are given under Expts. 3, 4 and 5. The meal contained protein 16·4 grams, fat 24·7 grams, carbohydrates 27·0 grams. Total calories 408. Ketogenic-anti-ketogenic ratio 0·75 : 1. Exp. 3 shows the simple experiment where no insulin was administered. That the patient was incapable of catabolising the available carbohydrate in the meal is shown by the fact that while the urine was sugar-free at the commencement of the experiment, the concentration of glucose at the end was 1·58 %. The effect of the meal containing all three principal food components is to raise the total metabolism 7 % above the level obtained with fat and protein alone. That this increase does not result from an increased combustion of the carbohydrate is shown by the fact that the rise in the total metabolism is not coincident with the highest respiratory quotient. One may therefore conclude that it is due to the promoting action of the carbohydrate on the combustion of protein and fat. The rise in the blood sugar level does not take place until the final specimen, when a value, of 0·27 % was obtained. This corresponds to the finding of sugar in the urine of 1·58 %. In spite of the ketogenic-anti-ketogenic ratio of the food being 0·75 : 1·0, the excretion of acetone bodies was increased after food, due doubtless to the inefficiency of carbohydrate utilisation.

Exp. 4 was done six days later and demonstrates the effect of 10 units of insulin upon the metabolism after the ingestion of protein, fat and carbohydrate. The basal metabolism on this morning was—21·0 % with a respiratory quotient of 0·71. There was a trace of sugar in the urine in spite of the fact that the blood sugar was 0·17 %.

The immediate effect of the injection of 10 units of insulin was to increase

the combustion of carbohydrate as shown by the elevation of the respiratory quotient from 0·71 to 0·735. The drop in the respiratory quotient as the result of giving food is well seen and the rise to a value of 0·785 is thereafter rapid. As the lowest respiratory quotient is coincident with the greatest oxygen absorption it is evident that carbohydrate can have taken little part in the elevation of the metabolism.

The amount of insulin administered was not sufficient to protect the patient from a characteristic rise in blood sugar, which reaches its highest point in the final blood sugar estimation 2½ hours after the patient received food. Whether insulin has an effect on the absorption of carbohydrate from the alimentary canal, which seems unlikely, or whether some step in the intermediary fate of the food stuff is accelerated, there appears to be a more immediate rise in the level of blood sugar with than without insulin, and this phenomenon we have seen in others of our experiments. As the commonly accepted ratio between the amount of sugar capable of being catabolised per unit of insulin used is as 2 : 1 and the food contained in all 39 g. of available carbohydrate from the bread, 9·52 from the protein, and 2·47 from the fat, the rise in blood sugar and increase in urinary sugar was to be anticipated with only 10 units of insulin given.

In order to see if an increase in insulin dosage would alter the type of curve, another experiment was performed seven days later (Expt. 5). The daily diet had been increased until the patient was receiving 1153 Calories per day, of which 56 Calories were contributed by carbohydrates. The same meal as in the previous experiment was administered, preceded, however, by 15 units of insulin. In this experiment the amount of insulin was sufficient to keep the blood sugar below the threshold for this case and there was, therefore, no sugar excreted in the urine. As will be seen, the trend of the total respiratory exchange and respiratory quotients is similar to that in the previous experiment, the respiratory quotient falling as the result of the administration of food. There is no late depression in total metabolism. Viewing this experiment as a whole it seems that the organism was able to utilise the greater part of 39 g. of carbohydrate. That the low respiratory quotients were not due to a retention of CO_2 by the liberated alkali on the disappearance of the ketonic acids from the blood, as Davies and his co-workers suggest, is negatived by the fact that the concentrations of acetone compounds in the urine at the beginning and end of the experiments are not in harmony with this idea. If large quantities of lactic acid were formed, there would be a tendency to an increased lung ventilation. There was, as a matter of fact, a decrease. The only theory therefore which appears to satisfy the condition is a transformation of the carbohydrate into glycogen or into a substance of unknown composition.

At this point in his treatment the patient received 15 units of insulin daily, administered in three 5-unit doses. His total consumption of food, 57·3 g. as protein and 37·6 g. as carbohydrate, was equivalent in all to 1471 Calories. After seven days of this treatment we estimated the effect of

three ounces of bread in one meal after protection by 20 units of insulin given in one dose (Exps. 6 and 7). As the bread given contained 40·5 g. of carbohydrate, 6·6 g. of protein, and 6·6 g. of fat, the total amount of available glucogenic material was 44·0 g. Assuming the customary ratio between carbohydrate and insulin we should have expected practically complete protection by the hormone. The results of giving the three ounces of bread preceded by an injection of 20 units of insulin were as follows.

Owing, doubtless, to his continued insulin treatment, the basal metabolism on this day was—8·0 %, 12 % higher than that obtained in the previous week. The respiratory quotient was 0·730, indicating a metabolism concerned chiefly with the combustion of protein and fat. The diet even with the amount of insulin given contained an excess of carbohydrate, as shown by the high blood sugar, and the presence of glucose in the urine. After the 14 hour fast the urine was sugar- and acetone-free.

The immediate effect of 20 units of insulin was to depress the total metabolism and elevate the respiratory quotient. This immediate depression of the total metabolism was not seen in the previous experiment with smaller dosages of insulin, but was seen in other of our experiments, notably case II, where 10 units were sufficient to depress the total metabolism. It is not altogether easy to explain this depression. Kellaway and Hughes suggest the transformation of carbohydrate to some substance unknown, the change involving an economy of oxygen and a liberation of excess of carbon dioxide. It is possibly also a protective phenomenon in which the proportion of carbohydrate burnt is increased and thus spares protein and fat. This involves a lower amount of oxygen used and hence is represented in indirect calorimetry as a fall in the total metabolism. It will be noted that during this period the respiratory quotient rose.

The ingestion of three ounces of bread raised the metabolism 9 % above the determined basal level, whereas protein and fat of the same caloric value decreased the level 17 %. The blood sugar rose almost immediately after the ingestion of bread to 0·265 %. With this figure the respiratory quotient rose in the course of the morning to 0·80, the highest obtained with the patient.

Twenty units of insulin in this patient depress the total metabolism and increase the proportion of carbohydrate utilised. The drop in the respiratory quotient after food is again pronounced and does not confirm the observation of Wilder, Boothby and Beeler [1922], who state that the depressor effect is greatest after protein.

Two days after the foregoing series of tests was performed, the daily dose of insulin was increased to 24 units. With this three ounces of bread were given, one after each injection. Sugar was still present in the urine. The dose of insulin was raised to 30 units per day, and finally to 45 units per day. With this dose the patient remained aglycosuric and added 11 pounds to his weight. The effect of giving three ounces of bread without the protection of insulin was now determined. It will be noted (Exp. 7) that the basal metabolism

had risen as the result of treatment from -20 % to $+3$ %, confirming Joslin's observations [1918] and lending support to the contention that the basal metabolism is really low in severe cases of diabetes.

In the present experiment the specific dynamic action of the carbohydrate followed on the rise in the respiratory quotient and indeed the total metabolism had already fallen when the respiratory quotient was still on the increase.

There does not seem to have been any increase in carbohydrate tolerance, for, referring to Exp. 3, it will be seen that 39 g. of available carbohydrate raised the blood sugar at the end of $2\frac{1}{2}$ hours to 0·270 %, while in the present experiment, the blood sugar rose to 0·266 % after three ounces of bread. There was undoubtedly a certain amount of carbohydrate utilised, as the respiratory quotient of 0·846 indicated, but the 20 units of insulin given did not produce a normal catabolism of sugar.

Having discussed in some detail the results obtained with one case of diabetes we now present the results of experiments with three others. These results will be discussed only in so far as they bear comparatively on the first case.

Case II, aged 50, was one with a marked family history of diabetes. The urine on admission contained 6·9 % of glucose; the blood sugar was 0·245 %. He reacted very quickly to fasting, the urine becoming sugar free in two days. Exps. 10 and 11 were performed on this patient. The results go to show that insulin by itself enables the reserve carbohydrate to be catabolised, and at the same time depresses the total metabolism, and furthermore lowers the dynamic action of a given meal when injected 30 minutes prior to the meal.

Case III is presented as that of a diabetic with a history of loss of weight extending over ten years. As will be seen from Exps. 14, 15, 16 and 17, this patient reacted less promptly to insulin. The elevation of the respiratory quotient from insulin alone was present but less marked. The depression of the specific dynamic action of protein was not seen. There was little increase in carbohydrate tolerance even after treatment with insulin for 45 days, showing the refractory nature of the condition. Case IV is presented as a contrast to the foregoing. The patient was a youth of 19 years of age, who had been treated in the hospital about eight months previously, during which time his tolerance had been raised so that he was able to take Graham's full diet with two ounces of brown bread and one ounce of potatoes. His urine at that time was free from sugar and the blood sugar was 0·086 %.

On re-admission the urine contained 4·2 % of glucose. After 25 g. of glucose the blood-sugar rose to 0·340 %. As will be seen from Exp. 18, the effect of insulin on this patient was very striking. It produced within 25 minutes an elevation of the respiratory quotient from 0·763 to 0·830, with a drop of 7 % in the oxygen absorption.

The administration of two ounces of bread increased the metabolic rate 13 % above the basal value. The stimulation of metabolism was only transitory.

In two hours, however, the respiratory quotient had risen to 0·890, indicating that a considerable quantity of carbohydrate was being oxidised.

The second experiment with this patient was a control of the foregoing. He received two ounces of bread without the preliminary protection with insulin. The effect of this is strikingly shown in Exp. 19. The basal oxygen absorption had dropped to − 12 % and the respiratory quotient was at the high level of 0·877. The blood sugar level had fallen to 0·114 %, the urine was sugar-free and contained only a trace of acetone. Obviously the patient was reacting promptly to the restricted diet which was that of Graham's third day.

Case III. Computed oxygen absorption in the basal state: *210 cc. per min.*

		Per min.				Litres	Blood	Urine %		
	Time	O_2 cc.	B.M.R. %	CO_2 cc.	R.Q.	lung vent.	% sugar	sugar	acetone	Remarks
Exp. 14	9.15	—	—	—	—	—	0·133	0	2·19	Absolute rest
	9.55	206	− 2	144	0·699	5·59	—	—	—	Absolute rest
	10.20	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 g., ingested
	10.55	226	+ 8	151	0·667	5·20	—	—	—	Patient quiet
	11.0	—	—	—	—	—	0·191	—	—	Patient quiet
	11.30	226	+ 7	166	0·735	5·50	—	—	—	Patient quiet
	12.0	242	+15	164	0·676	5·42	—	—	—	Patient quiet
	12.30	247	+18	170	0·687	5·75	—	—	—	Patient quiet
	12.40	—	—	—	—	—	0·195	2·76	2·16	
Exp. 15	9.15	—	—	—	—	—	0·083	0	1·90	Absolute rest
	9.55	210	± 0	142	0·677	4·78	—	—	—	Absolute rest
	10.0	—	—	—	—	—	—	—	—	*Insulin* U. 15
	10.25	208	− 1	144	0·690	4·95				
	10.30	—	—	—	—	—	0·112			
	10.35	—	—	—	—	—	—	—	—	P. 16·4 g., F. 24·7 g., C. 27 g., ingested
	11.15	235	+12	154	0·656	5·12	—	—	—	Patient quiet
	11.20	—	—	—	—	—	0·178	—	—	Patient quiet
	11.45	243	+15	175	0·719	5·45	—	—	—	Patient quiet
	12.40	248	+18	183	0·739	5·63	—	—	—	Patient quiet
	12.45	—	—	—	—	—	0·170	0	1·30	
Exp. 16	9.10	—	—	—	—	—	0·127	0	Trace	Absolute rest
	9.45	210	± 0	140	0·669	4·56	—	—	—	Absolute rest
	9.50	—	—	—	—	—	—	—	—	*Insulin* U. 20
	10.15	194	− 7	132	0·680	4·55	—			
	10.20	—	—	—	—	—	0·113			
	10.30	—	—	—	—	—	—	—	—	P. 6·6 g., F. 6·6 g., C. 40 g., ingested
	11.0	217	+ 3	144	0·680	4·74	—	—	—	Patient quiet
	11.10	—	—	—	—	—	0·210	—	—	Patient quiet
	11.30	239	+14	166	0·696	5·19	—	—	—	Patient quiet
	12.0	232	+10	160	0·692	5·00	—	—	—	Patient quiet
	12.30	234	+11	158	0·674	5·01	—	—	—	Patient quiet
	12.45	—	—	—	—	—	0·182	1·25	Trace	
Exp. 17	9.30	—	—	—	—	—	0·110	0	0	Absolute rest
	10.0	200	− 5	145	0·723	4·70	—	—	—	Absolute rest
	10.15	—	—	—	—	—	—	—	—	P. 6·6 g., F. 6·6 g., C. 40 g., ingested
	11.20	222	+ 6	166	0·749	6·18	—	—	—	Patient complained of pain on application of the nose clip
	11.30	—	—	—	—	—	0·230	—	—	Patient complained of pain on application of the nose clip
	12.15	218	+ 4	160	0·736	5·06	—	—	—	Patient complained of pain on application of the nose clip
	12.30	—	—	—	—	—	0·232	2·34	0	

Case IV. Computed oxygen absorption in the basal state: *223 cc. per min.*

	Time	Per min. O_2 cc.	Per min. B.M.R. %	Per min. CO_2 cc.	R.Q.	Litres lung vent.	Blood % sugar	Urine % sugar	Urine % acetone	Remarks
Exp. 18	9.10	—	—	—	—	—	0·210	0	Trace	Absolute rest
	9.50	215	− 3	164	0·763	4·75	—	—	—	
	10.0	—	—	—	—	—	—	—	—	*Insulin* U. 10
	10.25	200	−10	166	0·830	5·31				
	10.30	—	—	—	—	—	0·204			
	10.35	—	—	—	—	—	—	—	—	P. 4·4 g., F. 4·4 g., C. 27 g., ingested
	11.5	246	+10	192	0·781	5·31	—	—	—	
	11.15	—	—	—	—	—	0·246	—	—	Patient quiet
	11.35	221	− 1	174	0·790	5·38	—	—	—	
	12.0	211	− 5	164	0·777	5·21	—	—	—	
	12.30	209	− 6	186	0·890	6·19	—	—	—	
	12.45	—	—	—	—	—	0·244	0	Trace	
Exp. 19	9.20	—	—	—	—	—	0·114	0	Trace	Absolute rest
	10.0	195	−12	171	0·877	6·41	—	—	—	
	10.15	—	—	—	—	—	—	—	—	P. 4·4 g., F. 4·4 g., C. 27 g., ingested
	10.55	225	+ 1	167	0·741	6·05	—	—	—	
	11.0	—	—	—	—	0·184	—	—	—	Patient quiet
	11.30	217	− 3	164	0·756	5·59	—	—	—	
	12.0	212	− 5	166	0·783	5·72	—	—	—	
	12.30	211	− 5	165	0·783	5·35	—	—	—	
	12.45	—	—	—	—	—	0·240	Trace	Trace	
Exp. 20	9.15	—	—	—	—	—	0·122	0	Trace	Absolute rest
	10.0	197	−11	166	0·847	5·63	—	—	—	
	10.5	—	—	—	—	—	—	—	—	*5 pan-secretin tablets given*
	10.30	194	−12	164	0·845	5·18	—	—	—	
	10.35	—	—	—	—	—	0·118	—	—	P. 4·4 g., F. 4·4 g., C. 27 g., ingested
	10.40	—	—	—	—	—	—	—	—	
	11.30	224	+ 1	177	0·791	5·52	—	—	—	Patient quiet
	12.0	198	−11	160	0·807	5·35	—	—	—	
	12.45	201	−10	158	0·788	5·84	—	—	—	
	12.50	—	—	—	—	—	0·218	Trace	Trace	

The immediate effect of the food was most striking. The respiratory quotient fell to 0·741 and the oxygen absorption was increased. The specific dynamic action of the carbohydrate was only transitory, for by the end of the morning the oxygen absorption had fallen to − 5 %. The respiratory quotient did not rise to the high figure reached in the experiment with insulin, the quotient generally indicating a restricted use of sugar. The blood sugar curve is typically diabetic, the last point reaching the value 0·24 %. These experiments strongly suggest that the immediate effect of insulin when injected into a fasting diabetic is to depress the total metabolism and at the same time to increase the proportion of sugar oxidised. Probably the former is contingent on the sparing action of the carbohydrate. This, however, is not the only action of insulin, for the final high respiratory quotient reached after the injection of 10 units of insulin and not seen in the experiment without insulin can be explained by assuming that insulin enabled the organism to oxidise a greater proportion of sugar, not, however, immediately after the ingestion of this food, but after the stimulating effect of the carbohydrate on the total meta-

bolism had passed off. This delayed oxidation of sugar may be accounted for by assuming that the first action of insulin after the initial small combustion of sugar is to convert the carbohydrate into some intermediary complex and later to assist in its combustion.

Finally, a preliminary experiment is here reported on the effect of the oral administration of a preparation of the pancreas. The makers do not claim an antidiabetic action except after long continued administration, but it was hoped to demonstrate some effect As will be seen, Exp. 20, five "pan-secretin" tablets given in place of 10 units of insulin produced no immediate effect on the respiratory quotient, the total metabolism or the blood sugar percentage. There is no evidence of an increased utilisation of sugar due to "pan-secretin." Hence this product cannot be substituted for insulin when immediate results are to be anticipated.

Discussion.

The reaction to food and insulin depends on the age and the condition of the patients. The youngest patient of the series reacted quickly to a single dose of insulin. This case showed considerable capacity for oxidising carbohydrate 2½ hours after the injection of insulin. A long standing case of the disease as illustrated by Case III showed very little evidence of any ability to utilise carbohydrate better as the result of diet and insulin. It is notable that the basal metabolic rate of the former case increased as the result of treatment while the rate of the latter remained stationary. As the basal metabolic rate increased, as the result of treatment with diet and insulin, with no corresponding increase in weight of the patient, one may advance this fact in support of the idea that the basal metabolism is really decreased in diabetes apart from any consideration of loss of weight or decreased plane of nutrition. The tolerance of the first case showed a decided improvement during his stay in hospital, while the ability of the second case to utilise carbohydrate was not increased.

The presence and function of a small store of reserve carbohydrate is made evident in these experiments. It is to this store that the metabolism is directed by the injection of insulin. Immediately the reserve carbohydrate commences to be catabolised the protective action on the protein metabolism is manifested and the total respiratory exchange falls, more or less coincidently with a rise in the respiratory quotient.

Once the metabolism has been directed into more normal channels, the organism appears to attempt to preserve the store of carbohydrate and the protein and fat are now attacked. This is the second phase, evidenced by the return of the respiratory quotient to a lower level. In some instances this is paralleled by an increase in the total respiratory exchange.

The experiments do not bear out the theory of Davies, Lambie *et al.* [1923] that the delayed elevation of the respiratory quotient is due to a retention of carbon dioxide by the liberated alkali on the disappearance of the keto-acids

from the blood, for the curves of acetone excretion in the urine do not correspond with this. Nor do the experiments support the theory of Briggs and Koechig [1924], viz. that the action of insulin is to promote the conversion of glucose into lactic acid, for there is no substantial evidence of an increased lung ventilation, which we would anticipate if such were the case.

The observations seem to fit in rather with the more obvious explanation that the first action of insulin is to convert glucose into glycogen, and as soon as a reasonable demand of the tissue for this substance is satisfied, the insulin enables the organism to mobilise and burn the glycogen in the normal way.

The fact that Dudley and Marrian were unable to demonstrate an increased storage of glycogen in the livers and muscles of normal animals after treatment with insulin does not appear to disprove the theory that the first action of insulin is to convert glucose into glycogen or a similar substance and later to assist in its combustion, but rather tends to confirm it. In the normal animal there is already a sufficiency of glycogen in the tissues. The action of insulin in the normal would be to bring about a combustion of this glycogen rather than a polymerisation and storage of glucose. Hence the experiments on the normal animal only apply with reserve to the processes taking place in the diabetic.

We wish to tender our thanks to Dr E. Lloyd Jones and to Dr Aldren Wright for permitting us to investigate the cases in their service at Addenbrooke's Hospital; to the Sister of Griffith Ward for her unfailing assistance, and to the Medical Research Council for a grant to one of us, part of which has been used in the present work.

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XIX. THE INFLUENCE OF OXYGEN ON THE PRODUCTION OF UREA BY ENZYMES OF THE LIVER AND SPLEEN.

By ROBERT ALEXANDER McCANCE.

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(*Received November 11th, 1924.*)

In a previous paper, McCance [1924], the production of urea by mammalian spleen, liver and kidney in autolysis was described, and mention made of the marked inhibition of this reaction by performing the autolysis in pure oxygen gas. This inhibition has now been further studied. The general methods used to estimate the urea and regulate the p_H were described in the last paper and need no further comment.

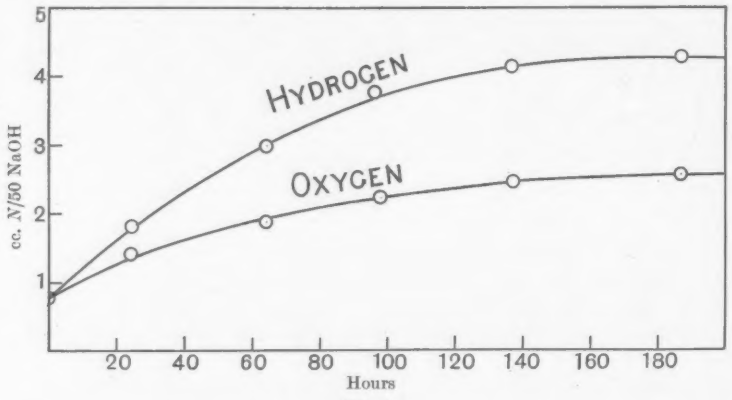

Fig. 1.

Fig. 1 shows the effect of oxygen on the production of urea by a spleen (ox) autolysing at p_H 6. The experiment was arranged as follows. Spleen was made up for autolysis at p_H 6, sampled, halved, placed in two filter flasks, and evacuated till free from air. One flask was then filled with oxygen, and one with hydrogen, well shaken, and both incubated at 37°. Each flask was evacuated and refilled with gas after sampling. Amounts of urea are expressed in cc. $N/50$ NaOH. Results such as this may always be obtained, the two curves ultimately becoming parallel to each other and to the time axis, showing that the total amount of urea produced is always much less in oxygen than in hydrogen.

The previous paper showed that the effect of oxygen gas was upon the enzyme, not upon the substrate. The following experiment (Fig. 2), which is one of several carried out, shows that while this oxygen effect is (largely at any rate) reversible, an autolysis begun in oxygen, even if subsequently changed to hydrogen, still shows a much smaller total production of urea than if kept in hydrogen gas from the beginning. The full curves indicate urea production in hydrogen, the dotted curves in oxygen. Autolyses of spleen at p_H 6 were set up exactly as described above in oxygen and hydrogen gas, and samples taken after one, and after two days. Each was then halved, and half the autolysis in oxygen was transferred after rapid and complete evacuation to hydrogen. In a similar way half the autolysis in hydrogen was transferred to oxygen. After the change over the two autolyses in hydrogen (one has been in hydrogen throughout, the other has been changed from oxygen to hydrogen) both show roughly parallel rates of production of urea. Thus, although the *activity* of the enzymes producing urea has not been much, if at all, reduced by the treatment with oxygen, yet the total *amount* of urea produced by enzymes so treated is always less than that of their fellows which have been kept in hydrogen throughout; and this although oxygen does not destroy the substrate.

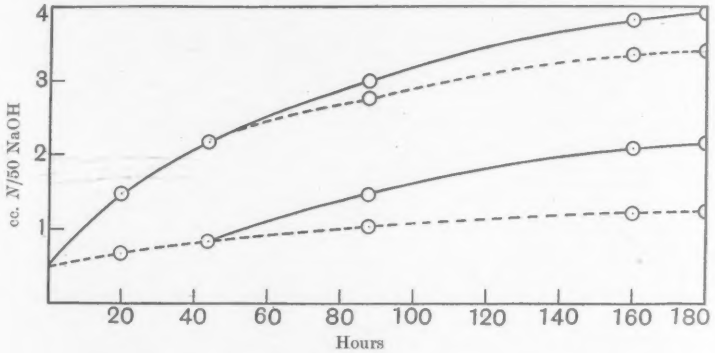

Fig. 2.

If the observations of the effect of oxygen gas on the production of urea are extended to autolysing liver, the results are different. At p_H 5 (where there is no formation of urea whatever in the spleen) the inhibition is slight but of essentially the same nature as that shown in Fig. 1, whereas at p_H 6 it may be, and usually is, completely absent, though there is a formation of urea.

Of quite a different nature are the curves in Fig. 3 showing the effect of oxygen on the production of urea by liver enzymes from the filtrate of a spleen completely autolysed at p_H 5. (As described in the previous paper, there is no formation of urea during the complete autolysis of spleen at p_H 5, but a very complete hydrolysis.) The procedure by which the curves were obtained

was as follows: 30 cc. of the autolysate, previously boiled, filtered, and titrated to p_H 7, were placed in each of two small filter flasks, evacuated, and filled, one with oxygen, the other with hydrogen. To prepare the enzyme suspensions, liver was made up as for autolysis at p_H 6, strained through muslin, and treated with oxygen and hydrogen exactly as described above in the case of the spleen. These remained 24 hours at room temperature, and were then incubated 14 hours at 37°. 3 cc. were then withdrawn from the suspension in hydrogen and added to the 30 cc. autolysate in hydrogen. Similarly 3 cc. from the suspension in oxygen were added to the autolysate in oxygen. To sample, 5 cc. were withdrawn at once, and again at suitable intervals, placed in boiling water, and estimated. After sampling, each flask was re-evacuated and refilled with gas. There is no formation of ammonia under these circumstances. The essential difference between the effect of oxygen gas as illustrated by Figs. 1 and 3 is that in Fig. 1 the oxygen gas reduces the rate of formation of urea and also the total amount formed, whereas in Fig. 3 the effect is only upon the rate, the total amount formed being ultimately the same in both oxygen and hydrogen. Fig. 3 completely bears out the conclusion, reached previously by a different method, that the oxygen does not exert an irreversible effect on the substrate.

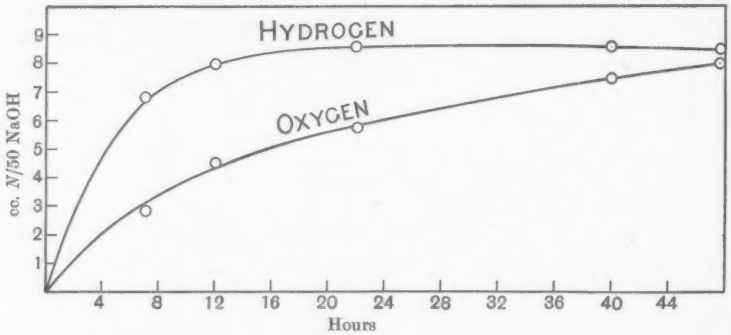

Fig. 3.

Only one explanation remains therefore of why the *total amount* of urea produced in an autolysis (Fig. 1) is less in oxygen than in hydrogen, although the effect of oxygen is only upon the velocity of urea production, and is reversible. The reactions giving rise to urea in such an autolysis come to an end, because the enzymes, whether in oxygen or hydrogen and apart from the influence of either gas, become inactive. This occurs before the enzymes treated with oxygen have time to decompose as much of their substrate as the enzymes treated with hydrogen. Two separate factors leading to this result have been discovered.

(1) The production of urea is greatly inhibited by the products of autolysis. While fractionating the products of a complete autolysis of spleen at p_H 5,

the whole of the precursor was found to be removed from solution by silver in saturated baryta solution, and it was noticed in studying the urea formation from this basic fraction how much more active the enzyme appeared to have become. The same effect was observed after a butyl alcohol extraction. The full curves (Fig. 4) show this inhibition due to products of autolysis. The dotted curve beside each full curve shows the inhibition due to oxygen (which is very slight in this particular case). These curves were obtained in the following way. The silver precipitation of Kossel was carried out quantitatively on the filtrate of a spleen completely autolysed at p_H 5. Both fractions were then evaporated to the original bulk.

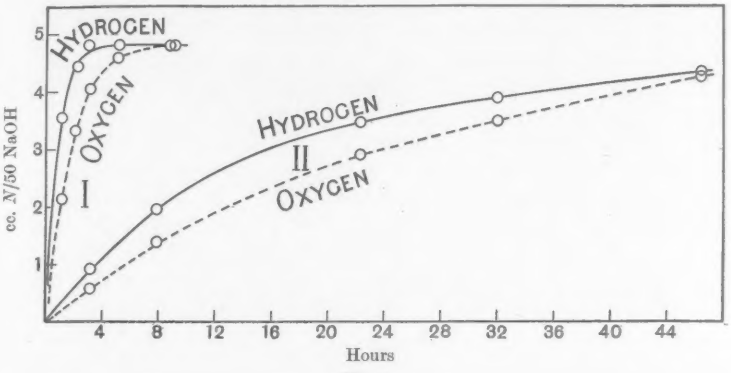

Fig. 4.

Curve I shows the urea production from the basic fraction; Curve II from the solution obtained by mixing equal volumes of both the basic and mono-amino fractions, and evaporating the whole to the volume of each before mixing. This is practically the composition of the original autolysate. The method of obtaining the curves has already been described in detail above. Both sets were of course taken at the same time with similar amounts of enzyme and under identical conditions. A theoretical meaning of these results may be that, granted that adsorption of the substrate on the enzyme precedes decomposition, many or all of the amino acids may also be adsorbed, and prevent access of the urea precursors to the active surface of the enzyme.

On applying these results to autolysis, it is evident, from the nature of the case, that this inhibition due to the products of autolysis must become greater and greater as autolysis proceeds, and so, other things being equal, the rate of production of the urea will become slower and slower.

(2) At the reactions used for studying autolysis, due mainly if not entirely to the p_H being an unfavourable one, the enzymes producing urea become more or less rapidly inactive. To investigate this, effects due to (1) must be eliminated. This was done when testing the activity of the enzymes by using a substrate containing such a high amino acid content that the comparatively

small increase of amino acids due to autolysis of the source of the enzyme during the experiment could be neglected. Such a substrate is spleen autolysed completely at p_H 5, boiled, filtered, and neutralised, which treatment gives almost as high a ratio $\frac{\text{Amino N}}{\text{Total N}}$ as is obtained by an acid hydrolysis. Liver, containing active urea-forming properties, was made up for autolysis at the p_H to be investigated, strained through muslin, and placed in the incubator at 37°, samples being withdrawn at once and again at suitable intervals, and the activity of the urea-producing enzymes tested. For this purpose 5 cc. substrate, 0·5 cc. of the liver suspension, and 10 drops of toluene were placed in a test-tube, and incubated 16 hours at p_H 7 and 37°, heated to 100° and the urea content estimated. Previous trial had shown that for the first 16 hours the rate of production of urea was approximately constant, reduction of rate due to lack of substrate not taking place till later. The activity of the enzymes producing urea falls off steadily from day to day, the rate depending on the p_H. Table I will make this more clear, the amount of urea formed in the 16 hours being taken as a measure of the activity of the enzymes, and their activity on the first day being reckoned as 100. Every test of activity was done in duplicate.

Table I.

p_H of autolysis, 6.

Days after setting up autolysis	Temp. of autolysis	Activity of the urea-producing enzymes
0	37°	100
2	,,	88
5	,,	76
8	,,	55

The steady fall in the activity of the enzymes shows that the effect is not due to accumulation of the products of autolysis in the liver suspension. Were it so, the rate of inactivation would be rapid at first and later fall off to a small figure.

The decline in the activity of the enzymes producing urea is much more rapid at p_H 5 than at 6, the activity being zero after three days. This is in keeping with the fact that the production of urea in liver autolysing at p_H 5 invariably ceases after three days. Even when freshly made up, the activity of the urea-producing enzymes of the liver made up at p_H 5 is never so great as that made up at p_H 6, though the p_H at which the activity is being studied is the same in both cases (p_H 7). The reason for this is probably the fact that phosphates accelerate the action of arginase [Gross, 1920], which certainly accounts for some of the urea formed under these conditions, and while phosphates were used to buffer the liver to p_H 6, phthalates were used for p_H 5.

It is now possible to define the conditions which must exist in order to obtain the inhibition by oxygen gas of the formation of the urea in autolysis, and to explain the apparent contradictions between the liver and the spleen results. The precursors of the urea must be set free by the enzymic hydrolysis

of the tissue proteins at a rate faster than they can be decomposed by the urea-forming enzymes when inhibited by oxygen gas. In other words, the factor limiting the velocity of urea-formation must be the activity of the urea-producing enzymes themselves. In the spleen this is ideally obtained about p_H 6. The protein hydrolysis is rapid and fairly complete; the enzymes producing urea are never very active in this organ, and at p_H 6 their power to form urea becomes progressively hampered by the accumulation of the products of autolysis, and the loss of activity due to the p_H. The result is that the autolysis in oxygen shows a smaller total production of urea than its control in hydrogen, the enzymes producing urea in oxygen becoming inactive before they have time to decompose as much of their substrate as their fellows in hydrogen. The liver on the other hand possesses urea-forming systems many times more active than those of the spleen. At p_H 6 the precursors of the urea are never set free fast enough to allow the inhibition by oxygen to become apparent, for even when inhibited by oxygen the production of urea keeps pace with the liberation of the precursor. The limiting factor is the rate of tissue hydrolysis, and therefore the rate of urea production and the amount of urea produced are the same both in hydrogen and oxygen in this case.

In the spleen autolysing at p_H 5, the conditions are so unfavourable for the production of urea (small concentration of enzyme, low p_H, very rapid hydrolysis of the tissue proteins with accumulation of end products) that the action of the enzymes producing urea never becomes apparent. But in the liver autolysing at p_H 5, the conditions are just right for the inhibition by oxygen to show itself. The factor limiting the formation of urea is the activity of the urea-producing enzymes as in the spleen at p_H 6. Their higher concentration is balanced by a correspondingly greater inhibition due to p_H.

Effect of combining the actions of urease and the urea-forming enzymes.

Spleen autolysed to completion at p_H 5, boiled, filtered, and neutralised was used as substrate, 10 % suspension of liver, of the mucous lining of a dog's stomach or of soya bean meal being used as the source of the enzymes.

Liver suspension plus urease acting together were always found to produce 25 % more urea (as NH_3) than liver acting alone. The tubes were incubated three days at 37° and after heating to 100° urease was added to each for 10 mins. at 40° before estimating. However, on arranging the experiment differently it was found that liver plus urease acting together for three days gave exactly the same amount of urea (as NH_3) as urease (alone) for three days *followed* by liver (alone) for three days. (After the first three days the urease was destroyed by boiling.)

This probably means that the substrate contains small amounts of the urea precursor still in a combined form which the proteolytic enzymes of crude soya bean meal and the mucous lining of a dog's stomach can liberate, while the liver, though unable to liberate the precursor, alone possesses the means of decomposing it.

SUMMARY.

(1) That oxygen gas markedly inhibits urea production in autolysis has been confirmed.

(2) The effect of oxygen is merely to inhibit the rate of urea production by tissue enzymes, but in autolysis where other factors (p_H, accumulation of amino acids, etc.) are in operation simultaneously, the effect of oxygen may also be to reduce the total amount of urea formed.

The author wishes to express his gratitude to Professor Hopkins for his advice and criticism.

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XX. THE INFLUENCE OF STORAGE ON THE ANTISCURVY VALUE OF FRUITS AND VEGETABLE JUICES.

By ELLEN MARION DELF.

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(*Received December 23rd, 1924.*)

In recent years, attempts to place our knowledge of food values on a wider scientific basis have led to a series of researches on the effects of cold storage on a variety of products. One of the earliest of these was an investigation started in 1918 at the Lister Institute which was organised by Dr H. Chick and assisted by a grant from the Food Investigation Board. The object of the experiments was to find out the effects of prolonged storage under various conditions on certain common fruit and vegetable juices known to be of considerable antiscorbutic value when fresh. The tests have been carried out from time to time by a number of workers, all following the same methods and with a considerable amount of co-operation. Preliminary results with chilled fruits and fruit juices stored at various temperatures were published by Davey from the Lister Institute in 1921. The continuation of the work was interrupted however owing to unforeseen circumstances and the results have not yet been presented in a collected form. The research begun at the Lister Institute by Davey and others was continued by me in 1923 at the Biochemical Department, Institute of Physiology, University College and in the following year at Westfield College, in the Department of Botany, with the help of a grant from the Food Investigation Board. I have to thank Prof. Drummond for his kindness in connection with the experiments at University College and Dr H. Chick for giving me free access to previous records at the Lister Institute and for kindly reading the proofs of this article.

The technique of testing the juices is that described in detail by Delf and Tozer [1918], following in the main that of Chick, Hume and Skelton [1918]. Young guinea-pigs weighing at first about 320 g. were fed on a basal diet of crushed oats and bran together with a ration of 60 to 90 cc. of cow's milk previously autoclaved at about 120° for one hour. The fruit juices to be tested were fed by hand as daily doses and the smallest dose which effected protection from scurvy over an experimental period of 90 days was taken as the minimal protective ration for comparative purposes.

I. Cold storage of fruits and vegetables or their juices.

(*a*) *The storage of chilled fruits and vegetables.*

Oranges, lemons, tomatoes and swede-turnips were selected for trial as being easy to obtain in quantity and also rich in their content of vitamin C. Only carefully selected sound material was used. Each specimen was wrapped in paper to prevent contact with its neighbour, packed in a crate and conveyed at once to a chilling room of the Union Cold Store, Red Bull Wharf, E.C., whose proprietors kindly placed a chamber in their establishment at the disposal of the Lister Institute for this research.

(1) *Oranges.* A number of selected Denia oranges were packed and placed in the chilling room (temp. 2·5–5·4°) on May 7, 1919. They were inspected in July and again in October the same year when feeding experiments were started. At this time 96 oranges were still available of which only 11 were sound. The worst fruit was used first, the more rotten parts being cut away and the juice of the remainder squeezed as required through muslin. It was shown by Davey [1921] that the minimum protective dose of this juice was equal to that of the fresh raw fruit, being only 1·5 cc. daily. The storage and partial decay of the fruit had thus not appreciably impaired the antiscorbutic value of the juice.

(2) *Lemons.* Lemons packed and placed in the chilling room about the middle of April 1919 were inspected in July and November of the same year and were finally removed and tested in January 1920. Their condition was worse than that of the oranges, none being sound at the beginning of the experiment. The juice of these was fed to the experimental animals without previous neutralisation and frequently unsound parts of the lemons were used so that the juice was often discoloured. The daily doses were 1·5 cc., 2·5 cc. and 5 cc. respectively. In every case severe scurvy resulted, 51 days being the longest time of survival; little if any protection was therefore received.

The conclusion drawn from these experiments was that the juice of oranges and probably also of lemons does not lose appreciably in antiscorbutic value as long as the fruit is fit for food, but that the fruit cannot be kept in the chilled condition for more than four or five months.

(3) *Swede-turnips.* On May 10, 1919, swede-turnips were procured, some from Covent Garden and some from Newcastle through the kindness of Prof. Hutchens. Each consignment was examined immediately on arrival, only sound roots being used for the chilling room. The swedes were inspected in July and again in October of the same year. On the latter date, only a dozen of the Covent Garden batch remained sound; those from Newcastle were all more or less rotten: a few however were brought to the Lister Institute and tests were begun on the following day. They were only continued for 50 days owing to the rapid decay of such swedes as remained and towards the end of the experiment juice from unsound parts had to be included in the doses fed to the animals. Two animals on a dose of 2·5 cc. developed severe scurvy, this amount being

a sufficient ration in the case of the fresh juice. Two animals, each receiving 5 cc., were chloroformed after 35 days owing to lack of further juice. One showed typical scorbutic lesions while the other was normal. This experiment is inconclusive with regard to the effects of "chilling" since on the second visit to the store, some of the roots were found frozen which probably accounted for the rapid decay of the tissues. It is also inconclusive with regard to the antiscorbutic value of the juice, since protection was not attained over any appreciable experimental period. However, the results are not inconsistent with the conclusion derived from the consideration of the chilled oranges and lemons.

(4) *Tomatoes.* On August 6, 1919, a similar attempt was made to preserve tomatoes in the chilling room of the Union Cold Store. These were in fine condition when taken; they were examined in October the same year, and seemed mostly sound; a few with dark spots were carefully isolated with folds of paper. Examined again in January 1920, all the fruit was found to be spoiled and no further attempt was made to repeat the experiment.

(*b*) *Storage of fruit and vegetable juices.*

For commercial purposes, fruit juices are often preserved by the addition of a small proportion of some substance such as potassium metabisulphite. At low temperatures, however, the juice may be kept without the addition of any preservative and early in 1919, experiments were started to determine the influence of prolonged cold storage on the juice of oranges, lemons and swedes respectively.

(1) *Lemon.* On March 27, 1919, a number of sound lemons were well washed, dried and squeezed, the juice being then passed through muslin and placed in small glazed earthenware jars of about 150 cc. capacity, which were then at once corked and sealed with wax. A number of these jars were immediately stored in a cold chamber of the Colonial Consignment and Distributive Co., Ltd., which was generously set aside by them for the purpose. The temperature was checked by means of an automatic recording instrument and only varied from -11 to $-14°$. When samples of the juice were withdrawn for testing, they were always found to be frozen.

This juice remained undisturbed until August 1920 when test experiments were started at the Lister Institute by Davey, a jar of the frozen juice being fetched every fortnight for the purpose. Two animals each receiving a daily ration of 1·5 cc. of this juice remained free from scurvy for 90 days. This is no more than the minimal daily amount of the fresh juice giving protection from scurvy; there was thus no appreciable loss of vitamin during the first 17 months of cold storage.

In February 1924 some of the remaining juice was tested, jars of juice being fetched once a week. Three animals each receiving 1·5 cc. of the juice early began to show typical symptoms of scurvy. When these became severe, the ration was increased to 2·5 cc. One showed no improvement and was

chloroformed after 38 days; the other two were given a further allowance (2·5 cc. for 12 days, 3 cc. for the last 51 days). At the post-mortem, all these showed typical scorbutic lesions, severe in the first case, and nearly healed in the case of the two on increased doses. Three other animals on a daily ration of 3 cc. throughout the experiment remained in perfect health and showed no sign of scurvy at post-mortem examination.

These experiments show clearly that during five years' storage in the frozen state, a slow deterioration takes place, a minimum ration of nearly 3 cc. being required for protection instead of 1·5 cc. the amount needed of the raw fresh juice. Of the 135 jars originally stored only 22 have been used, the rest being still buried under an accumulation of snow in the freezing chamber. By prolonging the curve B (Fig. 1) smoothly, it will be seen that if the deterioration continues in the same way, a dose of about 5 cc. given in 1926 (*i.e.* after seven years' storage) should just suffice for protection.

(2) *Orange.* On March 13, 1919, orange juice was prepared, sealed and taken to the same cold storage chamber as the lemon juice. The first tests were made by Davey beginning in August 1920. As in the case of the lemon juice, a daily dose of 1·5 cc. was found to give complete protection and no measurable deterioration had occurred after 17 months of cold storage.

In February 1924 further tests were carried out by the writer, after five years of storage. Three animals on the usual basal diet were each given daily doses of 1·5 cc. of the juice. After 40, 42 and 49 days respectively, symptoms of scurvy became defined; the doses were then increased until at the end of the whole period the average ration per day amounted to 2·5 cc. At post-mortem examination all were found to have enlarged rib-junctions and dark coloured muscles; and one had also small lesions in the tissues around the knees and rib-junctions.

Three other animals kept on a uniform ration of 3 cc. survived for 91 days in good condition, but with occasional symptoms of scurvy. At the post-mortem examination, two had somewhat brittle bones, with some of the rib-junctions beaded; the third had in addition small petechial haemorrhages over the rib-junctions of both right and left side. This dose may therefore be taken as marginal in value. From these results, it appears that this sample of orange juice is perhaps somewhat less stable than the lemon (Fig. 1, A, B).

(3) *Swede-turnip.* The swedes for this experiment were obtained, some from Covent Garden and some from Newcastle as for the storage of the whole roots by chilling. The swedes were washed, grated on an ordinary kitchen grater, and the resulting pulp squeezed through muslin. The juice was placed in small glazed earthen jars of about 70 cc. capacity and was stored with the orange and lemon juice on 14th May 1919.

In August 1920, after 15 months' storage, the frozen juice was tested at the Lister Institute by Davey. Two animals each receiving 2·5 cc. of the juice developed acute scurvy and were chloroformed in an advanced condition after 37 days. Two others each receiving 5 cc. developed symptoms of

scurvy on the 26th and 28th days respectively. Both were chloroformed after 35 days of experiment and the post-mortem examination showed typical signs of severe scurvy. Two other animals were given 10 cc. daily beginning in October 1920; both became very ill with scurvy complicated by intestinal trouble, and had obviously received no protection. The administration of larger doses of the juice was not practicable.

Swede juice thus differs materially from the juice of oranges and lemons in that after only 15 months of cold storage in the frozen condition practically no antiscorbutic value remains. Some of this juice remains still in the freezing chamber and might be worth comparison with the fresh juice from the chemical point of view.

Table I. *The relative antiscurvy value of fresh and stored frozen juices.*

	Minimum protective ration cc.	Loss of vitamin C
Orange, fresh, raw	1·5	
Stored, frozen:		
1 year 5 months	1·5	Not appreciable
5 years	>2·5	More than two-fifths
5 years	about 3	About half
Lemon, fresh, raw	1·5	
Stored, frozen:		
1 year 5 months	1·5	Not appreciable
5 years	>2·5	More than two-fifths
5 years	<3	Less than half
Swede, fresh, raw	2·5	
Stored, frozen:		
1 year 3 months	> 5	Much more than half
1 year 5 months	>10	„ „ three-quarters

In the series of experiments described above, uniformity of material was secured as far as possible by the choice of ripe sound fruit, and by testing samples of the same material before and after storage. This is of some importance, since it is known that the antiscurvy value of the fresh product is itself subject to a good deal of variability. It has been proved for instance that the antiscorbutic value of orange juice diminishes somewhat towards the end of the season [Davey, 1921, p. 89], and that young carrots are more effective than old ones [Hess and Unger, 1919]. How far these and other variations in the condition of the fruit also affect the keeping properties is almost entirely unknown. Recent researches on the cold storage of apples show that in at least one variety, the time of survival in cold storage varies greatly, according to the age of the tree, the orchard and the season in which the crop was grown [Haynes, 1924].

The striking contrast between the behaviour of orange or lemon and swede juice calls for further comment. An obvious difference lies in the acidity. Acid fruit juices are found to be considerably more stable than neutral ones as regards antiscorbutic properties. Recently Zilva [1923, p. 416] has found that when decitrated concentrated lemon juice has part of its acid restored

there is much less loss of antiscorbutic properties on storage. According to La Mer and Campbell [1921] and La Mer [1921] the degree of acidity in tomato juice also affects its stability when exposed to rise of temperature. However, different juices of about the same natural acidity may have very different degrees of stability in their antiscorbutic properties, *e.g.* swede juice is more stable to heat than cabbage juice, and orange juice nearly neutralised as tested by Delf [1920] is much more stable than tomato juice neutralised to about the same extent as described by La Mer [1921].

In the preparation of the orange, lemon and swede juice for storage, no attempt was made to exclude air and slow oxidation doubtless occurred. Onslow has recently shown that in many fruits (apple, plum, pear) the cell sap contains enzymes and their substrates forming an autoxidising system which is incomplete in the case of orange, lemon and various other acid fruits. It is possible therefore that the greater destruction in the swede juice may be due to some kind of autoxidation in the juice, but there is no direct evidence as yet upon this point.

II. Storage of canned fruits (oranges and tomatoes).

(*a*) *Oranges.* In April 1919, a number of Denia oranges were peeled, divided into convenient parts and packed into cans[1]. These were processed for 20–30 minutes, the temperature rising gradually to 100° and remaining at 100° for not more than five minutes. The cans were then sealed, cooled and stored at laboratory temperature. Five months later, the first test was made. Allowing for the small amount of water added to the fruit after packing the cans, a dose equivalent to 1·5 cc. of the fresh juice was found to give adequate protection from scurvy. No loss of antiscorbutic activity was therefore indicated at this time.

A further test was made after four years' storage, beginning on June 6, 1923. Three animals each receiving daily 3 cc. of the juice escaped from scurvy, but at post-mortem examination the muscles were of a very dark colour, and in two cases small periosteal haemorrhages were seen in the regions of the costochondral junctions. These doses were measured without allowance for the water added in canning and represent 1·5–1·7 cc. of the original juice. Animals on a higher ration (equivalent to 2·5 cc.) were completely protected from scurvy. The canned orange juice has thus lost but a small proportion of its original antiscurvy value, after storage for more than four years. If the minimum protective ration be plotted against the time of storage, it appears that the canned juice stored at laboratory temperature is more stable than the raw juice kept at $-10°$ to $-14°$. In view of this result, it seems probable that swede juice could also be successfully stored if previously heated and sealed in absence of air. This has only a theoretical interest, but it would

[1] The first batch was prepared by Miss Campbell; the rest by myself; the testing was done by myself.

probably apply equally to fruits such as pears and plums which are frequently bottled but the antiscorbutic value of which is little known.

(*b*) *Tomatoes.* The effect of storage on canned English tomatoes has been recently described [Delf, 1924]. The minimum protective dose of the raw juice lies near 1·5 cc. (estimated at about 2 cc.); that of the freshly canned tomato was about 8·5 cc.[1], this deterioration being in marked contrast to that which occurred with orange juice. After storage for four years, a dose averaging 10 cc. daily gave almost perfect protection for 68 days. For the full experimental period of 90 days a slightly increased ration would probably have been needed. The deterioration caused by storage is slow but is greater than that in the case of heated orange juice (Fig. 1). The reason for this is not clear since both are acid fruits and were canned and stored in the same way. As a matter of fact, it was noticed that the tomato juice when removed from the tins on opening did not keep as well as the orange juice especially in the hot weather. For the most part, juice showing signs of fermentation was rejected, but owing to the small supply fresh tins of canned tomatoes could not be opened as frequently as desired especially towards the end of the experiment.

Table II. *The relative antiscurvy value of freshly canned and stored canned fruit.*

	Minimum protective ration	Loss
Orange juice:		
Fresh, raw	1·5 cc.	
Canned, newly	1·5 cc.	Not perceptible
Canned and stored 4 years at lab. temp. ...	1·7 cc.	About 10 %
Tomato:		
Fresh, raw	1·5–2·5 cc. Estimated at about 2 cc.	
Canned, newly	7·5–10 cc. Estimated at about 8·5 cc.	About 75 %
Canned and stored 4 years at lab. temp. ...	>10 cc.	Over 80 %

III. Storage of raw lemon juice with added preservative.

In 1918 a quantity of lemon juice was prepared for storage by the addition of potassium metabisulphite as described by Davey [1921].

(*a*) *Material preserved with* ·06 % *sulphite.*

Two lots of this material were made up. The first made in February 1918 was tested after four and again after six months, but the results were not satisfactory owing apparently to exposure to air during the intervals of the experiment, and are set aside for the purpose of the present discussion. A further test on the second lot of material was made at intervals varying from 18 to 22 months. The experiments were not quite finished at the time

[1] According to Hess [1921] and La Mer [1921] bottled tomato juice in America has the much lower minimum protective ration of 3 cc.; the juice used by La Mer was prepared by a well-known canning company and was processed for a much shorter time than that prepared and used at the Lister Institute.

when the account by Davey went to press, but apparent protection was given by doses of 2·5 and 5 cc., no post-mortem examination being made in either case. In 1923 a further test was made by me at University College. No special precautions were taken to exclude air from the stock bottle which was kept at laboratory temperature throughout the experiment. No deterioration seemed to occur either in the appearance or taste during this time.

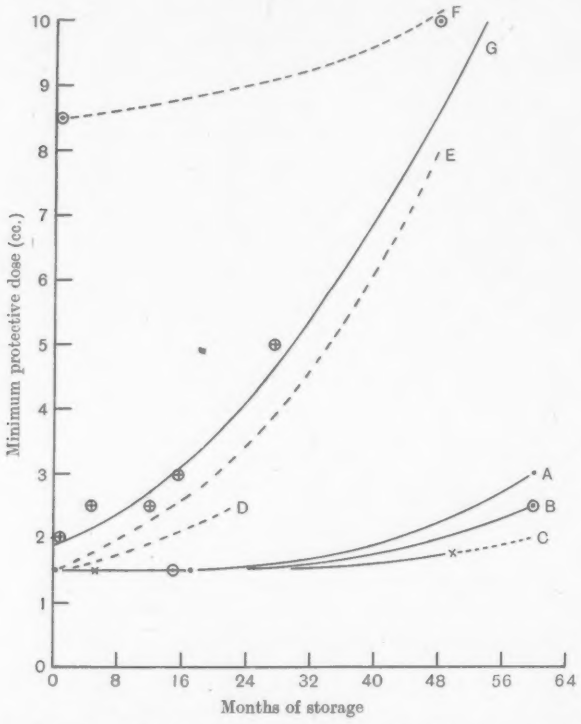

Fig. 1. Diagram showing relation between time of storage and minimum protective ration.

(A) Frozen orange juice (·—·), data as in Table I.
(B) Frozen lemon juice (⊙——⊙), data as in Table I.
(C) Canned orange juice (×—×), data as in Table II.
(D) Sulphite lemon juice ·06 % (cold room), data as in Fig. 2, D.
(E) Sulphite lemon juice ·06 % (lab. temp.), data as in Fig. 2, E.
(F) Canned tomato juice (⊙- - - - - -⊙), data as in Table II.
(G) Lemon juice with rind oil (lab. temp.) (⊕ — ⊕), data as in Table III.

Note. The curves D and E are taken from those in Fig. 2 of the same lettering, where the amount of approximation is shown on the same scale.

Three strong young guinea-pigs were selected for the purpose of the test. They disliked the juice so much that for the first 12 days the ration was kept

at 5 cc., after which it was increased to 10 cc.[1] fed in two daily instalments with other food between. The animals made fairly good growth and escaped typical scurvy but often showed signs of tenderness in the hind limbs. At post-mortem examination, after 88 days of experimental diet, one of the three was found to be quite normal; one had a haemorrhage into the wall and cavity of the middle region of the small intestine and the third had two small periosteal haemorrhages near the costochondral junctions. In every case the lower molars and in one case the tibiae also were markedly brittle. This dose, averaging 9 cc. daily over the whole period, therefore gave apparent protection, which, as seen from the post-mortem, was very nearly marginal. The undoubted value of the juice in this case suggests that this sulphite sample stored at laboratory temperature had a considerable degree of stability. It is obvious, however, that it is inferior in this respect either to frozen juice (Fig. 1, cp. E, B) or to the same juice stored at a lower temperature (Fig. 1, D).

A sample of this preparation was also stored at about + 4° and was examined after 27 months [Davey, 1921].

The results of these and other experiments are collected in Table III. Assuming the process of ageing to be an orderly one and making allowances for the approximate nature of some of the results, they may be expressed graphically (Fig. 2). The general form of the curve E is defined by the determination of the minimum dose after 4½ years' storage; the other points are less well known, but unless there was any initial rapid loss of antiscorbutic value on adding the preservative (a point which was not investigated at the time) the course of deterioration might be expected to follow some such direction as the line suggested in the smoothed curve E. The curve F is not so well defined since the final test failed to give protection; from what has been said about the relative value of the other doses, it cannot be very different from that indicated and the continuation of it must at least indicate a rate of deterioration considerably greater than that of the juice with a smaller amount of added preservative (curve E). This is clearly in opposition to the tentative conclusion of Davey [1921, p. 98] that the larger amount of preservative was probably to be recommended.

(*b*) *Material preserved with* 0·09 % *sulphite.*

Results of the earlier experiments with juice having a final concentration of 0·09 % added sulphite were briefly indicated by Davey [1921], but the experiments were unfinished at the time of publication. After seven months' storage, a daily dose of 5 cc. gave protection but no lower dose was then tried. After 12 months, three animals on a dose of 2·5 cc. received partial protection, symptoms of scurvy becoming well defined but not developing until the end of the experiment. The three animals on this dose behaved differently, one

[1] The average dose for the whole period was 9 cc., but since the lower ration was fed only at the beginning of the experiment, the result may be taken as due to a ration throughout of about 10 cc.

dying with severe scurvy after 61 days, another showing no symptoms until after 66 days and the third having no symptoms until after 94 days. In the case of the last animal the scurvy was of a definite but mild type although the experiment was prolonged for 100 days.

After 19 months' storage, a daily dose of 5 cc. gave considerable protection to each of two animals. The first developed symptoms after 78 days and died with severe scurvy after 84 days of experiment; and the second developed symptoms after 84 days and survived for 90 days, showing signs of mild scurvy at post-mortem. A dose of 10 cc. afforded complete protection in an experiment begun when the juice was 22 months old and continued for 86 days.

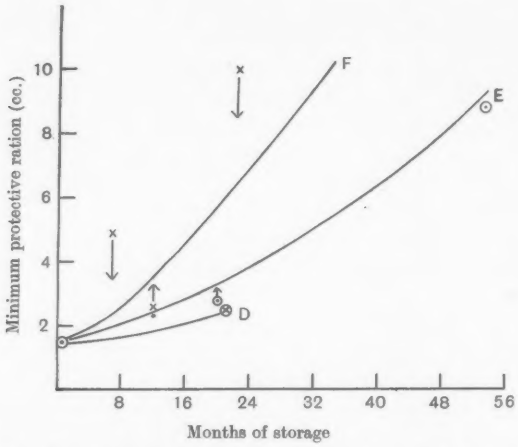

Fig. 2. Diagram showing the relation between time of storage and probable minimum protective ration; lemon juice with added sulphite:

·06 % stored at laboratory temperature, (⊙ ⊙) E.
·06 % stored in cold room (4–10°), (⊗ ⊗) D.
·09 % stored at laboratory temperature, (× × ×) F.

In June 1923 an attempt was made by me to apply a further test, after $4\frac{1}{2}$ years of storage at laboratory temperature. Three animals were started on a daily dose of 5 cc., but owing to difficulties of feeding the ration was not further increased. Symptoms of scurvy were noticed on the 52nd, 53rd and 67th day respectively. The symptoms were progressive, the last animal surviving for only 80 days, when it was chloroformed in an advanced stage of severe scurvy. No larger doses were practicable, but a consideration of all the results seems to show that the higher percentage of sulphite is not conducive to the preservation of the antiscorbutic value of the juice (cp. Fig. 2).

IV. The use of rind oil as a preservative.

The fresh juice of lemons and oranges as prepared with the rind oil by Davey in 1918 was stored part at laboratory temperatures[1] and part in the cold room of the Lister Institute. The results are given by Davey [1921] and are summarised for reference in Table III. In the summer of 1923, a further test was made using a daily ration of 5 cc. This gave partial protection to each of three animals which developed "chronic" scurvy, becoming severe but not fatal. It was impossible to feed larger doses at the time but some indication of the course of deterioration may be seen by plotting all the values found (Fig. 1, G).

Table III. *Lemon juice with added preservative.*

Condition	Temp.	Age	Minimum protective dose	
Raw	Laboratory	Fresh	1·5 cc.	
With ·06 % sulphite	,,	18–22 months	?>2·5–<5 cc.	Fig. 2, E
	,,	4½ years	9–10 cc.	
	Cold room	27 months	<5 cc.	Fig. 2, D
			>2·5 cc.	
With ·09 % sulphite	Laboratory	7 months	< 5 cc.	Fig. 2, F
		12	> 2·5 cc.	
		19	> 5 ,,	
		22	<10 ,,	
		4½ years	> 5 ,,	
With rind oil	Laboratory	Fresh	>1·5<2·5 cc.	Fig. 1, G
		4½ months	<2·5 cc.	
		12	<2·5 ,,	
		27	5 ,,	
		5 years	>5 ,,	

A similar set of experiments over a shorter period was carried out using orange juice. The stored orange juice behaved in much the same way as the lemon juice [Davey, 1921] but there is not sufficient evidence to make a detailed comparison of the stability of the two juices.

General conclusions.

(1) When whole fruits (oranges or lemons) or tuberous roots (swedes) are stored, their antiscorbutic value is retained as long as the tissue itself remains living.

(2) The expressed juice of oranges and lemons stored for five years in the frozen condition retains about half its original antiscorbutic potency. Swede juice on the other hand stored in a similar way, loses the greater part of its value in less than two years. It is suggested that the greater deterioration in the latter case may be due to the lower acidity or to autoxidation or to both.

(3) Canned orange and tomato juice retain much of their value even after storage for four years at laboratory temperature. The tomato as prepared for these experiments was much less stable than the orange.

[1] For the first 10 months all the juice was kept in the cold room; later part was removed and stored at laboratory temperature.

(4) Lemon juice with added potassium bisulphite retains its flavour well after storage for $4\frac{1}{2}$ years at laboratory temperature. At this age, the protective dose of lemon juice containing ·06 % sulphite was about 10 cc. instead of the original 1·5 cc., thus indicating a loss of about five-sixths of the original value.

(5) Both orange and lemon juice can be preserved satisfactorily for one to three years at laboratory temperature in presence of their own rind oil.

(6) Inspection of the comparative curves in Fig. 1 shows the relative stability of the juices examined. Canned orange is the most stable and this is in agreement with previous findings with heated juices [Delf, 1920]. The frozen juices are next in order of stability; the form of the curves in preserved juices suggests that the added sulphite has a destructive effect on the stability of the antiscorbutic vitamin.

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XXI. A CHEMICAL STUDY OF DEFECTIVE OSSIFICATION IN RACHITIC ANIMALS.

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Any attempt to understand the mechanism of ossification leads naturally to a consideration of those conditions which give rise to abnormalities in bone development. Apart from their intrinsic interest the investigation of such cases may throw light on the process as it takes place in the normal individual and it was for such reasons that the present study of ossification in rachitic animals was commenced.

Among the very large number of published papers on the cause and nature of rickets, relatively few deal with the chemical aspects of the defective ossification, the immediate cause of the failure to deposit the normal amount of calcium salts in the rachitic bone. Some fairly complete analyses of the inorganic constituents of normal and rachitic bones have been published, and the concentration of calcium and phosphate in the blood has been studied by a number of investigators. While their evidence shows that in children and in animals, rickets is frequently associated with a lowered content of inorganic phosphate in the blood, this is not always the case and is not specific of the condition [Hess and Unger, 1922]. There is little evidence of any significant variation in the concentration of total calcium [Howland and Kramer, 1921]. It would seem that the investigation must go deeper if this complex chemical problem is to be solved.

On the basis of the suggestions put forward in previous papers [Robison, 1923; Robison and Soames, 1924[1]] the deposition of calcium phosphate in the growing bone would be adversely affected by any of the following factors:

[1] *Correction.* In our previous paper [Robison and Soames, 1924] the equation (1) on p. 752 (and the same equation on p. 749) should read

$$[Ca^{++}]^3 \times [PO_4^{=}]^2 = K_1[Ca_3(PO_4)_2].$$

The limiting value of $[H^+]$ below which $Ca_3(PO_4)_2$ would be deposited (p. 752) is $K_2\sqrt{\frac{k_2^3}{k_1[HPO_4^{=}]}}$.

The conclusion that this value depends on the respective solubility products and the dissociation constant of the acid phosphate ions is not affected although for this particular case it would also depend on the relative amounts of calcium and phosphate in solution. The case is, however, a hypothetical one for, as previously stated, the solid phase is neither $CaHPO_4$ nor $Ca_3(PO_4)_2$ but a complex basic phosphate of whose ionisation we have no certain knowledge.

1. Lowering of the concentration of calcium ions in the plasma either as the result of a diminution in the total calcium content or of a disturbance in the equilibrium between the ionised and un-ionised portions thereof.

2. Lowering of the concentration of the phosphate ion corresponding with the bone phosphate (probably hydroxyapatite), either as a result of a diminution in the total inorganic phosphate or of a shift in the equilibrium between the various phosphate ions (change of p_H).

3. Failure of the cartilage and bone cells to secrete the enzyme.

4. Deficiency in the supply of the phosphoric ester which is normally present in the blood and is hydrolysed by the bone enzyme.

5. Alteration in the permeability of the corpuscular membrane, capillary wall or cartilage.

6. Failure of the mechanism (if such exists) by which the p_H of the tissue fluid is raised within the cartilage.

The occurrence of either (1) or (2) unaccompanied by an equivalent rise in the concentration of the other ion would result in the product of the ionic concentrations falling below the solubility product (k) of the bone phosphate and the plasma becoming unsaturated with respect to this substance. Deposition of the phosphate might still occur if the local increase in the inorganic phosphate resulting from the enzymic hydrolysis of the phosphoric ester were sufficient to raise the product of the ionic concentrations above k, but the amount of the calcium salt deposited would be reduced.

Failure either of the enzyme or of the specific phosphoric ester would result equally in deficient calcification since the necessary local increase in the phosphate concentration would not occur.

The phosphoric ester is present chiefly in the corpuscles and the conditions under which it passes into the plasma and cartilage fluids are not fully understood, but it is clear that the supply of this ester to the site of ossification might fail owing to changes in the permeability of the membranes (of the corpuscles etc.) even though the total amount present in the whole blood was not reduced.

It is conceivable also that changes in permeability of the cartilage or of the capillary walls might, by affecting the Donnan equilibrium, alter the relative concentrations of calcium and phosphate ions in the plasma and cartilage fluid.

The participation of the bone enzyme in the ossification process has been supported by a considerable weight of evidence but the further suggestion of a possible mechanism by which the p_H is locally raised is a teleological speculation and arises mainly from the evidence of the high optimum p_H of the enzyme. No direct proof of such a mechanism has yet been found. Its effect would be to increase the activity of the enzyme and, independently of this, to favour the deposition of calcium phosphate and of calcium carbonate.

It is obvious that a deficiency either of calcium or of phosphate in the diet, resulting in a lowered concentration of these substances in the blood, will

adversely affect calcification of the skeleton and may be a contributory cause of this defect in rickets, but the evidence at present appears to show that in well marked cases of rickets the concentration both of total calcium and of inorganic phosphate in the blood may be normal. A considerable proportion of the calcium is certainly present in un-ionised forms and will play only an indirect part in determining the precipitation of calcium salts. The investigation of the relation between the ionised and un-ionised calcium in plasma is a difficult but very attractive problem which we have not touched in the present paper but are hoping to attack later.

The inorganic phosphate is probably very largely ionised but the concentration of the particular anion corresponding with the solid phase (bone phosphate) must in any case be very small. It will be affected chiefly by the p_H of the fluid but to some extent also by the various cations present.

The bones of rachitic rats have been shown to contain at least as much enzyme as normal bones [Robison, 1923] and have been used to demonstrate the deposition of calcium phosphate *in vitro*, by immersing them in solutions of phosphoric esters. Failure to calcify is certainly not due to lack of enzyme. A deficiency in the amount of the specific phosphoric ester was however an attractive possibility and its investigation formed the chief part of the work here described.

Experimental.

Effect of Diet.

The first series of experiments was planned and carried out early in 1923 in collaboration with Dr Goldblatt before it was known that the organic acid-soluble phosphorus compounds in blood consist of at least two esters, only one of which, the smaller fraction, is hydrolysed by the bone enzyme [Kay and Robison, 1924].

A considerable number of rats was divided into groups and fed on various diets deficient in the fat-soluble factor, calcium or phosphorus or in two of these constituents, normal controls being provided for each group. When growth had ceased or rickets developed the animals were bled and killed under ether anaesthesia and estimations of the inorganic phosphate and total acid soluble phosphorus in the whole blood were carried out by the Briggs modification of the Bell-Doisy method. The fact that the inorganic phosphate is increased *in vivo* during ether anaesthesia and *in vitro* when the blood is laked with water was not discovered until after these experiments were completed [Martland and Robison, 1924] and it is very probable that the values obtained were all about 10 % too high. The averages for each group should however be comparable. The values for the total P should be correct. The results are shown in Table I. Except in the case of those rats which were fed on the McCollum diet there is no evidence that the deficiency of the fat-soluble factor results in a lowered concentration of inorganic phosphate in the whole blood

or that the addition of cod-liver oil to such a diet causes any appreciable increase. The average organic acid-soluble phosphorus is, however, regularly increased by the addition of cod-liver oil to the diet, although the amount of the increase is small and within the limits of variation found in individual rats on the same diet.

The rats fed on the McCollum diet No. 3143 showed an abnormal behaviour in this respect. The inorganic phosphate level was markedly below that found with any other of these deficient diets but was raised by nearly 70 % when cod-liver oil was given, although the total P did not change. The values obtained are not unlike those found by other investigators using this diet.

The low availability of the phosphorus derived from the cereals in this diet is well shown by comparison of the results obtained with those of Group IV in which the animals received a much smaller amount of phosphorus but chiefly in the form of inorganic phosphate, derived from the marmite. The concentration of inorganic phosphate in the blood was here nearly normal and was not increased by the addition of cod-liver oil to the diet.

Diets.

F. Heated casein, starch, cotton seed oil, marmite, lemon juice, salt mixture, mixed to a stiff paste with distilled water.

G. Same as F, with omission of calcium from the salt mixture.

E.W. Cooked egg-white replaced casein in diet F and a salt mixture free from phosphate was used.

McCollum 3143. Wheat, maize, wheat gluten, gelatin, calcium carbonate, sodium chloride, mixed to a stiff cake with distilled water.

Table I.

Group	No. of rats in group	Diet	% of Ca and P in diet as given to animals		Acid-soluble P compounds in 100 g. whole blood		Histology
			Ca	P	Inorganic mg. P	Organic mg. P	
I a	11	F (−A)	0·25	0·54	8·2	26·7	Mild rickets or osteoporosis
I b	11	F + C.L.O.	0·25	0·54	8·2	29·3	Normal with slight osteoporosis in some cases
II a	5	G (−A − Ca)	0·04	0·59	8·4	26·0	One normal, others with osteoporosis or osteomalacia
II b	3	G, + C.L.O. (−Ca)	0·04	0·59	8·5	28·4	Normal or osteoporosis
III a	5	E.W. (−A, −P)	1·08	0·08	4·5	20·5	Mild rickets or osteoporosis
III b	3	E.W. + C.L.O. (−P)	1·08	0·08	4·3	24·7	Nearly normal
III c	3	E.W. + C.L.O. + phosphate	1·08	0·54	5·8	27·3	Nearly normal
IV a	4	McCollum 3143	0·76	0·19	3·1	19·0	Rickets mild and florid
IV b	3	McCollum 3143 + C.L.O.	0·76	0·19	5·2	17·0	Nearly normal, some osteoporosis
IV c	3	McCollum 3143 + phosphate + C.L.O.	0·76	0·42	7·5	29·7	Nearly normal

Soon after these experiments were completed it was found that only a fraction (about 20 %) of the phosphoric esters is hydrolysable by the bone enzyme and it became apparent that increases (2–3 mg. P) such as were observed when cod-liver oil was added to the diets might, if occurring entirely in this fraction, represent very significant changes. A further series of experiments was therefore planned in which the amount of phosphoric ester hydrolysable by the bone enzyme should be estimated according to the method

Table II.

			Acid-soluble P compounds in 100 g. blood			Analysis of bones			
No. of exp.	No. of rats	Diet	Inorganic mg. P	Organic mg. P	Hydrolysed by bone enzyme mg. P	H_2O %	Ca % of dry bone	P % of dry bone	Ca : P
1	6	McCollum 3143	2·5	20·1	3·6				
	5	Do + C.L.O.	4·1	20·2	4·2				
9	3	McCollum 3143	2·6	24·9	5·1	62·5	12·11	5·40	2·24
	3	Do + C.L.O.	4·6	21·1	3·1	56·6	16·17	7·44	2·17
16	2	McCollum 3143	3·2	19·3	3·2	—	14·5		
	2	Do + 10 cc. milk	5·1	18·8	3·2	—	16·2		
18	2	McCollum 3143	2·1	24·3	4·5	54·8	16·30	7·22	2·26
	2	Do + oxidised C.L.O.	5·4	27·7	3·9	47·8	18·37	8·46	2·17
3	3	F	5·1	27·0	3·4	47·2	16·46	7·72	
	2	F + irradiation	5·1	27·6	4·4	44·6	20·36	9·27	
	2	F + C.L.O.	5·6	27·3	4·6	40·8	21·95	10·64	
5	4	F	5·1	26·6	5·6	47·5	18·69	8·68	
	2	F + irradiation	5·3	25·4	5·3	44·9	21·06	9·75	
	3	F + C.L.O.	5·3	28·0	6·4	39·3	21·60	9·92	
12	6	F	6·5	30·3	10·0	45·4	16·38	7·59	2·16
	6	F + C.L.O.	4·9	32·5	8·2	39·2	21·13	9·71	2·18
13	4	F	7·0	27·3	7·6	46·5	15·95	7·42	2·15
	2	F + C.L.O.	5·4	34·0	7·8	39·6	20·48	9·45	2·17
14	4	F_1	4·6	29·5	5·9	41·5	19·73	9·18	2·15
	4	F_1 + C.L.O.	4·9	34·3	6·0	40·5	21·08	9·87	2·14
	4	F_1 + sodium phosphate injections	4·3	32·1	6·5	45·6	19·54	9·15	2·14
	4	F_1 sodium glycerophosphate injections	4·7	29·5	5·2	42·4	20·21	9·36	2·16
15	2	F	4·5	28·2	5·8	46·7	19·06	9·00	2·12
	2	F + butter fat	4·6	30·5	5·3	41·4	20·86		
	2	F + oxidised C.L.O.	4·4	31·3	5·7	39·1	21·32	9·84	2·17
17	2	F	3·8	24·0	5·4	54·4	16·91		
	2	F + unsaponifiable matter of C.L.O.	4·5	28·0	4·6	46·6	19·80		
8	2	E.W. + casein	5·9	30·1	6·3	39·3	17·12		
	2	Do + C.L.O.	5·7	35·0	7·1	39·7	18·61		
	2	Do + 5 g. spinach	5·8	26·9	7·2	41·0	19·86		
	2	Do + 5 g. cabbage	5·9	37·8	6·6	40·0	19·05		
11	4	F	8·6	27·6	4·9	—	15·3		
	3	F + 0·1 g. spinach	8·8	31·1	4·4	—	15·9		

Diet F_1 (Exp. 14) contained 0·54 % of calcium and 0·54 % of phosphorus but was otherwise the same as diet F.
Diet E.W. + casein (Exp. 8) contained 0·67 % of calcium and 0·73 % of phosphorus.
The calcium and phosphorus contents of the other diets are given in Table I.
In Exp. 16 the 10 cc. milk increased the calcium content of the diet by about 10 % and the phosphorus content by about 30 %.

described by Kay and Robison [1924]. Rats from the same litter and in most cases from the Lister stock were used and the males and females were divided as equally as possible between the groups. Three experiments were also made on guinea-pigs but were not very satisfactory and the results have been omitted from the Table. The animals were killed in fasting condition[1] by severing the carotid and jugular without anaesthetic and the blood was taken directly into 1 % trichloroacetic acid to stop the action of the phosphoric esterase in the blood. This precaution, however, introduced a new difficulty. The cloudiness which occasionally appears on addition of the ammonium molybdate solution in estimating phosphate by the Briggs method, became more frequent and intense. The cause was finally traced to the presence of protein cleavage products formed by the action of a pepsin-like enzyme in the blood. The hydrolysis occurs much more rapidly in 1 % trichloroacetic acid than when the blood is laked with water. We continued to use the 1 % trichloroacetic acid for laking but added the further quantity of the acid as soon as possible to precipitate the proteins. This difficulty was only encountered in the case of rat's blood. Human blood and that of rabbits appear to contain much less "pepsin." The results of these experiments are set out in Table II.

Inorganic phosphate. As in the previous series the concentration of inorganic phosphate in the blood of the rats on diet 3143 was abnormally low but was raised almost to normal by the addition of cod-liver oil. The figures for the groups receiving other diets containing a sufficiency of inorganic phosphate provide no evidence that the deficient calcification of the bones produced in absence of the fat-soluble factor is associated with an abnormally low concentration of inorganic phosphate in the blood or that this concentration is raised by cod-liver oil or other sources of the vitamin. The figures for the normal controls show considerable variations and are obviously affected by other factors of which we have insufficient knowledge.

Organic phosphoric esters. In the groups on diet 3143 the total amount of phosphoric esters was again not raised by cod-liver oil except in one experiment (18) in which the rats were exceptionally well grown, being older than usual when placed on the diet. On the other diets the total amount of phosphoric esters was increased by the addition of cod-liver oil, by oxidised cod-liver oil, by the unsaponifiable fraction of the oil and by cabbage. Against the conclusion that this increase is associated with the improved calcification of the bones must be placed the fact that in Exp. 3 the amount of the increase was vanishingly small although the improvement in the calcium content of the bones was very marked. On the other hand, in Exp. 11 a decided increase in the amount of the esters was accompanied by very slight improvement in the bones. Irradiation appeared to have little effect on this value.

As to the phosphoric ester which is hydrolysable by the bone enzyme, we can find from these figures no satisfactory evidence of any abnormally low concentration in the blood of rats on −A diets nor of any significant

[1] Except in Exp. 14, in which the rats were killed two hours after feeding and injections.

regular increase connected with the addition of cod-liver oil to the diet. Whether the increase in the amount of the other ester which is not hydrolysable by the bone enzyme may have any indirect effect on the calcification of the skeleton we are not at present prepared to offer an opinion. Possibly the investigation at present in progress of the phosphoric-esterase of the blood may throw some light on this question. The activity of this enzyme appears to be subject to very delicate control by the p_H of the blood and to influence in a very marked degree the inorganic phosphate level [Martland, 1925].

Effect of injecting sodium glycerophosphate.

As an additional method of discovering whether the defective calcification is due to a lack of the specific phosphoric ester it was further proposed to study the effect of injecting sodium glycerophosphate into rats on a diet deficient in the fat-soluble factor. Unfortunately, owing to the fact that the experiment (Exp. 14) was carried out during the summer months with rats from a particularly strongly growing stock, the controls failed to exhibit very marked symptoms and the percentage calcification of the bones was not greatly below that of the normal animals. Two litters of rats, divided into four groups each of four animals, were placed on the experimental diets when 21 days old, and 10 days later the injections of sodium glycerophosphate (1 cc. *M*/5 solution daily, subcutaneously) were commenced in one group. A second group received injections of equivalent amounts of sodium phosphate (Na_2HPO_4) while a third received no injections. The fourth group received cod-liver oil (by mouth) from the beginning to the end of the experiment which lasted 57 days. Analyses were made in duplicate of the bones of the hind legs of all animals of each group collectively and the blood was also investigated (Table II). The average Ca content of the dry bones of the groups receiving sodium glycerophosphate injections was 2·4 % higher than that of the groups receiving none and 3·4 % higher than that of the group receiving sodium phosphate injections which was even lower than that of the controls. Cod-liver oil increased the calcium content of the bones by 6·8 % above that of the −A group. The evidence of this experiment, that the injection of sodium glycerophosphate produces some increase of calcification while that of sodium phosphate has no such effect, could not, in view of the small increases observed, be considered satisfactory without further confirmation. We did not, however, continue these experiments as the subject was being very thoroughly investigated by Korenchevsky and Carr [1925]. As the result of their experiments they conclude that sodium glycerophosphate has a definite effect in increasing the calcium content of the bones of such rachitic rats, while injections of sodium phosphate produce less or no improvement. They find however that calcium glycerophosphate produces a much greater improvement than the sodium salt.

If we may draw any conclusion from these results and from our own, it is that while a deficiency of the specific phosphoric ester is not the cause of

the defective calcification, an excess of this ester may go some way towards counteracting the lack of the unknown responsible factor. The marked effect of calcium glycerophosphate suggests but does not prove that the deficiency is in the calcium ions.

That the sodium salt of the phosphoric ester possesses some beneficial effect not shown by sodium phosphate may be taken as additional evidence of the participation of the bone enzyme in the process of calcification.

The Ratio of Ca *and* P *in the bones.*

Both the phosphorus and calcium were determined in the bones of the experimental animals as it was thought possible that variations in the ratio Ca : P might throw light on the deficient factor. This ratio would be 1·93 for $Ca_3(PO_4)_2$, 2·15 for calcium carbonato-phosphate $(Ca_3P_2O_8)CaCO_3$ or for hydroxyapatite $(Ca_3P_2O_8)_3Ca(OH)_2$ (Bassett), and 2·37 for a mixture of 1 mol. hydroxyapatite with 1 mol. $CaCO_3$ (9 %). If the carbonate and phosphate are precipitated independently a deficiency of the phosphate ion might give rise to bones showing a higher ratio of Ca : P if the factors governing the precipitation of $CaCO_3$ were unaffected. A deficiency of the Ca ion would not necessarily alter the ratio, unless the calcium were replaced by magnesium. Calculations based on the published analyses did not furnish very hopeful results. Thus Gabriel's [1894] analyses give ratios of 2·27 for human bones, 2·24 for ox bone and 2·19 for goose bone; Gassmann's [1910] analyses of bones of normal and rachitic children give 2·24 and 2·16 respectively, the lower ratio in the rachitic bone being accounted for by an increase in Mg from 0·1 % to 0·64 % of the dry bone. Our own analyses were carried out in duplicate on aliquot portions of solutions obtained by igniting the bones of hind legs with nitric and sulphuric acid. The Ca was estimated by the method of Aron and Sebauer and P by that of Neumann. It would have been better had the bones first been extracted with alcohol and ether but it was found by actual experiment that the amount of P thus extracted does not represent more than 0·2 %–0·3 % of the total P of the bone so that the error introduced in the ratio Ca : P is very small. In Exps. 3 and 5 the Ca and P estimations were done on separate bones and the ratios are therefore of no significance.

In the experiments in which the basal diets, *F* and F_1, were not deficient in phosphate the ratios show a considerable degree of constancy, all lying close around 2·15 (± 1 %). There is a general tendency for the ratio to be slightly lower in groups on −A diet than in those receiving cod-liver oil. The ratios for the groups receiving McCollum's low phosphorus diet 3143 are decidedly higher (2·25) but are reduced to the normal figure by cod-liver oil. Without wishing to press these figures too far it may be said that they would accord with the supposition that in rats fed on diet 3143 the deficiency is chiefly one of phosphate ions while on diets deficient only in the fat-soluble factor it is the concentration of Ca ions which falls below the normal.

In such a complex problem as that of rickets we do not wish to suggest that our conclusions have necessarily any wider application than for the particular cases investigated, *i.e.* defective calcification produced in rats by

diets deficient in the fat-soluble factor. It is hoped to extend the investigations to the blood of other animals and of children in rachitic conditions and some data have already been obtained in this direction, but the need of a closer study of the various forms of calcium in the blood seems to be even more clearly indicated.

We are greatly indebted to Dr M. Boas, Dr H. Chick and to Professor Drummond for allowing us to investigate the blood of the animals in certain of their experiments (Exps. 8, 11, 16, 17) and for permission to quote their analyses of the bones of these animals.

SUMMARY.

The blood of rats fed on diets containing a sufficiency of calcium and inorganic phosphate but deficient in the fat-soluble factor contains a normal amount of inorganic phosphate and of the phosphoric ester hydrolysable by the bone enzyme, and these are not increased by the addition of cod-liver oil to the diet.

The amount of the phosphoric ester resistant to the action of the bone enzyme is usually increased by cod-liver oil or other sources of the fat-soluble factor but there appears to be no direct connection between this increase and the improvement in the calcification of the bones.

The defective calcification of the skeleton resulting from such diets is probably not due to any deficiency of inorganic phosphate or of the specific phosphoric ester in the blood, or of the enzyme in the bone by which the ester is hydrolysed.

An excess of hydrolysable esters (caused by the injection of sodium glycerophosphate) may however partly counteract the lack of the unknown responsible factor and produce some improvement in the calcification of the bones.

In rats fed on a diet (McCollum 3143) very deficient in inorganic phosphate the concentrations of inorganic phosphate and of phosphoric esters in the blood are below the normal. The inorganic phosphate level is raised almost to the normal by cod-liver oil but the amounts of the esters are not significantly increased.

The ratios of Ca : P in the bones support the view that in rats fed on this diet the defective calcification is due mainly to a deficiency of inorganic phosphate ions while this is not the case when the defect is caused by a lack of the fat-soluble factor alone.

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XXII. AN ARITHMETICAL TEST OF THE VALIDITY OF THE THEORY OF BAYLISS REGARDING FERMENTATION AND ADSORPTION.

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On page 315 or 316 in the various editions of Bayliss' *Principles of General Physiology*, a table is given, which is reproduced below, in which the values of the "specific activity" of the ferment trypsin when added in different concentrations to a solution containing equal quantities of caseinogen are shown, as determined by experiment.

Relative concentration of enzyme (1)	Time taken for equal change in minutes (2)	Mean rate × 1000 (3)	"Specific Activity" (4)	Theoretical values (5)
8	41	24	3	3
5	48	20·8	4·16	3·9
4	55	18·2	4·55	4·55
2	81	12·4	6·2	6·1
1	144	7	7	7

Bayliss remarks upon this table:

"The reader will probably notice at once that these results are precisely what we should expect if the velocity were controlled by adsorption; it was, in fact, this relationship which first led me to suggest the hypothesis of adsorption as applying to the case."

Neither Bayliss, however, nor his present editors seem to have thought it worth while to test arithmetically whether the values obtained by the experiments are consistent with the usually adopted formula of adsorption.

Mathematically this is a piece of elementary algebra though the arithmetic is lengthy. The adsorption formula is usually written in the form,

$$x = \alpha C^{\frac{1}{n}} \quad \ldots\ldots\ldots\

If the concentration of the substance, in the present instance trypsin, be originally m, this may be written in the form

$$x = a\,(m - x)^{\frac{1}{n}} \quad \text{.....(2).}$$

It is convenient, as $\frac{1}{n}$ is a fraction, to raise both sides to the power n. This is due to the fact that the unknown is contained in the expression on the right-hand side of the equation, not by itself but combined with a known but varying quantity m. The form of the equation used in calculation is thus

$$x^n = a^n\,(m - x) \quad \text{.....(3).}$$

If the amount of adsorption, as is assumed in Bayliss' theory, be taken as proportional to the rate of action, column (3) in the table, the equation can be easily solved. It is convenient for purposes of calculation to divide the figures in the third column of the table by seven making the lowest figure unity. With this notation, taking the first, third and fifth terms of column (3) and comparing with the corresponding amount of enzyme, column (1), we have the three equations

$$\left(\tfrac{24}{7}x\right)^n = a^n\left(8 - \tfrac{24}{7}x\right) \quad \text{.....(4),}$$

$$\left(\tfrac{18{\cdot}2}{7}x\right)^n = a^n\left(4 - \tfrac{18{\cdot}2}{7}x\right) \quad \text{.....(5),}$$

$$x^n = a^n\,(1 - x) \quad \text{.....(6).}$$

The solution is obtained by dividing equations (4) and (5) by equation (6) and then eliminating x. The eliminant equation after reduction has the form

$$68{\cdot}6\left(\tfrac{24}{7}\right)^n - 224\left(\tfrac{18{\cdot}2}{7}\right)^n + 347{\cdot}2 = 0 \quad \text{.....(7).}$$

From this equation n has to be determined. This was solved by taking a series of values of n, namely, 2, 3, 4, and 5. The root having been located between $n = 4$ and $n = 5$, a series of approximations resulted in the final value of 4·1723. This gives $\frac{1}{n} = {\cdot}240$, a number in accord with all the values experimentally found to hold in adsorption experiments.

Using the values of n it was directly calculated that the value of a^n was equal to 22·84 and of x equal to ·973. From these the values of the "specific activity" for $m = 5$ and $m = 2$ were evaluated and are given in the fifth column of the Table. The chief difference which exists is that in the second line between the values 3·9 and 4·16. A second approximation distributing the error over all the observations would give a closer fit but as the labour of solving such equations is considerable, and as the fit is all that could be desired from the experiments, it does not seem worth the trouble. The calculation clearly demonstrates that with trypsin and caseinogen, the experiments are consistent with the theory that the action of a ferment is in accordance with the adsorption formula as generally accepted.

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